Atrial fibrillation risk loci interact to modulate Ca\(^{2+}\)-dependent atrial rhythm homeostasis

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Atrial fibrillation (AF), defined by disorganized atrial cardiac rhythm, is the most prevalent cardiac arrhythmia worldwide. Recent genetic studies have highlighted a major heritable component and identified numerous loci associated with AF risk, including the cardiogenic transcription factor genes TBX5, GATA4, and NKX2-5. We report that Tbx5 and Gata4 interact with opposite signs for atrial rhythm controls compared with cardiac development. Using mouse genetics, we found that AF pathophysiology caused by Tbx5 haploinsufficiency, including atrial arrhythmia susceptibility, prolonged action potential duration, and ectopic cardiomyocyte depolarizations, were all rescued by Gata4 haploinsufficiency. In contrast, Nkx2-5 haploinsufficiency showed no combinatorial effect. The molecular basis of the TBX5/GATA4 interaction included normalization of intra-cardiomyocyte calcium flux and expression of calcium channel genes Atp2a2 and Ryr2.

Furthermore, GATA4 and TBX5 showed antagonistic interactions on an Ryr2 enhancer. Atrial rhythm instability caused by Tbx5 haploinsufficiency was rescued by a decreased dose of phospholamban, a sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor, consistent with a role for decreased sarcoplasmic reticulum calcium flux in Tbx5-dependent AF susceptibility. This work defines a link between Tbx5 dose, sarcoplasmic reticulum calcium flux, and AF propensity. The unexpected interactions between Tbx5 and Gata4 in atrial rhythm control suggest that evaluating specific interactions between genetic risk loci will be necessary for ascertaining personalized risk from genetic association data.

Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia, affecting more than 7 million Americans and 33 million people worldwide (1). AF is characterized by an irregular pattern of atrial depolarization, resulting in rapid and disorganized atrial conduction and lack of effective atrial chamber contraction. The rhythm abnormality in patients with AF manifests with circulatory deficits and systemic thromboembolism that greatly increase morbidity and mortality. Because age is an independent risk factor for AF, its prevalence is expected to rise significantly as the population ages. AF has become a major clinical and economic burden, owing to the limitations and side effects associated with current AF therapies. Although AF most often manifests in the context of pre-existing cardiac pathologies, such as hypertension and cardiomyopathy, idiopathic or lone AF forms have indicated a heritable component (2). Genome-wide association studies (GWAS) have to date identified more than 100 AF-associated loci, including many transcription factor (TF) loci, suggesting that transcriptional control of atrial rhythm is an important mediator of AF risk (3, 4).

Recent work has illuminated a role for abnormal cardiomyocyte calcium (Ca\(^{2+}\)) handling in the cellular pathophysiology of AF. The current paradigm of AF causation describes ectopic (triggered) atrial activity, mediated by early and delayed afterdepolarization (EAD and DAD) events and a fibrilligenic substrate that propagates the abnormal triggers causing arrhythmia. EADs and DADs have been associated with abnormal cardiomyocyte Ca\(^{2+}\) handling, including RYR2 dysfunction, reduced sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (encoded by ATP2A2) activity, and/or increased Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) activity (5–9). It remains to be defined how alterations of RYR2, ATP2A2, or NCX expression in AF models contribute to AF risk. GWAS and familial inheritance have implicated transcription factor genes in AF pathogenesis, including the cardiogenic transcription factors (TFs) TBX5, GATA4, and NKX2-5 (2, 10). Autosomal dominant mutations in the T-box TF TBX5 cause Holt-Oram syndrome, characterized by upper limb malformations, congenital heart defects, cardiac conduction system abnormalities, and increased AF risk (11, 12). In addition, GWAS has identified common risk variants associated with PR interval and increased AF susceptibility in intergenic or intronic regions of TBX5 (13–16). Adult-specific Tbx5 deletion in mice leads to spontaneous and sustained AF, characterized by slowed conduction and decrements in the expression of ion channels linked to AF (17). TBX5 drives the atrial expression of Pitx2, and Tbx5 and PITX2 codominate the expression of cardiac rhythm effector genes, including Ryr2 and Atp2a2. These findings indicated that interactions between TBX5 and PITX2 provide tight control of an atrial rhythm gene regulatory network and that perturbation of this network triggered AF susceptibility (17). This example suggested that cardiac TFs implicated in AF by genetic association may coregulate a gene regulatory
network for atrial rhythm homeostasis. GATA4 and NKX2-5 are particularly relevant candidates, given that they both interact physically and genetically with TBX5 during cardiac development. GATA4, a zinc finger transcription factor, plays critical roles in heart development and cardiomyocyte differentiation (18–20). Several GATA4 loss-of-function mutations have been reported to underlie AF susceptibility in humans (10, 21–23). GATA4 is highly expressed in adult cardiomyocytes; however, its specific role in atrial cardiac rhythm has not been investigated. NKX2-5, a homeodomain containing TF has been implicated in AF by GWAS (13). Heterozygous mutations in NKX2-5 are associated with a spectrum of congenital heart diseases (CHDs) in humans and mice (27–31). NKX2-5 also has been shown to regulate a number of target genes involved in the cardiomyocyte action potential, suggesting that it may play a direct role in AF predisposition (32).

Combinatorial interactions between TBX5, GATA4, and NKX2-5 are critical for heart development. TBX5, GATA4, and NKX2-5 physically interact, and CHD-causing (but not CHD-sparing) mutations in TBX5 abrogate these interactions (33). Mutations in GATA4 that disrupt transcriptional cooperativity with TBX5 result in CHD and impaired cardiac gene expression, leading to aberrant chromatin states and gene expression (33, 34). Tbx5/ Gata4 double-heterozygous mice develop cardiac defects that are more severe than Tbx5 or Gata4 haploinsufficient mice alone, providing evidence for a cooperative interaction (35). TBX5, NKX2-5, and GATA4 synergistically activate multiple cardiac enhancers and promoters (33, 36–45). Cooperative interactions between TBX5, NKX2-5, and GATA4 on cardiac gene expression may rely on interdependent binding of these factors genome-wide, enabling co-regulation of the cardiac differentiation program (46). Removal of Tbx5 or Nkx2-5 in mouse embryonic stem cell cardiac differentiation or GATA4 in humans induced pluripotent stem cell cardiac differentiation resulted in inappropriate distribution of the other TFs to lineage-inappropriate sites, inducing ectopic gene regulation (34, 46). This paradigm suggested that heterotypic interactions between these cardiogenic TFs may influence atrial rhythm control.

We investigated the combinatorial genetic interactions between the cardiogenic transcription factors Tbx5, Gata4, and/or Nkx2-5 in murine atrial rhythm control. We hypothesized that adult-specific combined Tbx5, Gata4, or Nkx2-5 haploinsufficiency may alter AF susceptibility and illuminate AF pathophysiology. Surprisingly, we found that Gata4 haploinsufficiency rescued the cardiac rhythm, cardiomyocyte electrophysiology, and molecular defects caused by Tbx5 haploinsufficiency. In contrast, introducing Nkx2-5 haploinsufficiency had no observed effect on the penetrance or severity of defects caused by Tbx5 haploinsufficiency alone. Gata4 haploinsufficiency rescued the calcium-handling defects and Ryr2 and Atp2a2 gene expression deficits caused by Tbx5 haploinsufficiency. We found that GATA4 negatively modulated TBX5 activation of a cis-regulatory element at Ryr2. This observation indicated that GATA4 could repress TBX5-dependent transcriptional activation in some contexts and provided a molecular model for the rescue of Tbx5 haploinsufficiency by Gata4 haploinsufficiency. We tested the hypothesis that rescue of Tbx5 haploinsufficiency was mediated by rescue of calcium homeostasis. Introduction of haploinsufficiency for phospholamban (Pn), a SERCA inhibitor, into Tbx5-haploinsufficient mice caused normalization of cardiac rhythm and rescue of SERCA activity. Rescue of the Tbx5-haploinsufficient phenotype by Gata4 deficiency illuminated a TF genetic interaction in atrial rhythm control and provided a molecular model for transcriptional control of cardiomyocyte calcium flux as a central component of atrial rhythm homeostasis.

Results

Reduced Gata4 rescues atrial arrhythmias caused by reduced Tbx5. We previously demonstrated that adult-specific Tbx5 deletion or haploinsufficiency caused spontaneous AF or AF susceptibility, respectively, linking TBX5 dose with AF risk (17). GATA4 and NKX2-5 physically and genetically interact with TBX5 during cardiac development, and all 3 cardiogenic TFs are strongly expressed in the adult atrium and are genetically linked to AF risk in humans (Figure 1A and refs. 2, 33, 35, 41, 46–48). We therefore hypothesized that GATA4 and NKX2-5 would genetically interact with TBX5 in adult atrial rhythm control. We employed a conditional knockout strategy to establish haploinsufficiency of Gata4, Tbx5, and Nkx2-5 singly or in combination in the adult mouse, affording normal gene dosage throughout development to circumscribe early lethality or structural heart defects observed with each mouse model (43, 49–51). We combined TF floxed alleles (Gata4fl/+; Tbx5fl/+; Nkx2-5fl/+; R26CreERT2) with a tamoxifen (TM)-inducible Cre recombinase allele at the Rosa26 locus (R26CreERT2) to generate adult compound haploinsufficient Gata4/Tbx5 (Gata4fl/-; Tbx5fl/-; R26CreERT2), Gata4/ Nkx2-5 (Gata4fl/-; Nkx2-5fl/-; R26CreERT2), Tbx5/Nkx2-5 (Tbx5fl/-; Nkx2-5fl/-; R26CreERT2), and triple-haploinsufficient (Tbx5fl/-; Nkx2-5fl/-; R26CreERT2) mice (28, 43, 52). All TF allelic combinations were generated and evaluated as littermates in a mixed genetic background. Mice were treated with TM at 6 weeks of age and loss of Gata4, Tbx5, and/or Nkx2-5 expression was confirmed in the left atrium by qPCR 2 weeks following TM treatment (Figure 1B and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI124231DS1). Specifically, Gata4 decrements were not observed in Tbx5 or Nkx2-5 single heterozygotes, indicating that TBX5 or NKK2-5 alone does not regulate expression of Gata4 (Figure 1B and Supplemental Figure 1A). Similarly, Tbx5 (or Nkx2-5) expression is not regulated by GATA4 or Nkx2-5 (or Tbx5) alone. However, a greater reduction in Gata4 expression was observed in Tbx5/Nkx2-5 compound heterozygotes compared with their respective single-haploinsufficient controls (P = 0.195 vs Tbx5fl/+; R26CreERT2, P = 0.038 vs Nkx2-5fl/+; R26CreERT2, respectively), suggesting that Tbx5 and Nkx2-5 may cooperatively regulate Gata4.

We first confirmed that atrial rhythm was sensitive to Tbx5 dosage in these mixed-background crosses by examining adult Tbx5 heterozygotes (Tbx5fl/+; R26CreERT2). Significant prolongation of the P-wave duration representing atrial depolarization (AF-associated finding in human studies) was observed in Tbx5fl/+; R26CreERT2 mice compared with control littermates 2 weeks after TM treatment by conscious ambulatory telemetry ECG (P = 0.0008) (Figure 1C and Supplemental Table 1). The PR interval, representing the period between initiation of atrial and ventricu-
**Figure 1. Gata4 haploinsufficiency rescues atrial arrhythmias caused by Tbx5 haploinsufficiency.** (A) Common and rare gene variants in the transcription factors TBX5, GATA4, and NKX2-5 have been linked to increased AF susceptibility. (B) Relative transcript expression by qPCR in the left atrium of Tbx5fl+/R26CreERT2, Gata4fl+/R26CreERT2, and Gata4/Tbx5 compound heterozygotes 2 weeks after TM treatment. Data are represented as means ± SEM normalized to GAPDH and relative to R26 CreERT2 mice (set as 1) \((n = 7–8\) R26 CreERT2, \(n = 6–7\) Tbx5fl+/R26CreERT2, \(n = 9–10\) Gata4fl+/R26CreERT2, \(n = 12\) Gata4fl+/Tbx5fl+/R26CreERT2). Experiments were performed in technical duplicates. \(P\) values were determined by 1-way ANOVA followed by Tukey post-hoc test. **\(P = 0.01\); ***\(P = 0.001\); ****\(P = 0.0001\). (C and D) P-wave duration and PR interval calculated from ambulatory telemetry ECG recordings from R26CreERT2 \((n = 6)\), Tbx5fl+/R26CreERT2 \((n = 8–9)\), Gata4fl+/R26CreERT2 \((n = 6)\), and Gata4fl+/Tbx5fl+/R26CreERT2 \((n = 10)\) mice. Tbx5fl+/R26CreERT2 adult mice displayed significant increase in P-wave duration (C) and prolongation of the PR interval compared with R26CreERT2 littermate controls (D). \(P\) values were determined by 1-way ANOVA followed by post-hoc Tukey test. (E-H) Intracardiac atrial electrogram recordings and corresponding surface ECG of R26CreERT2 \((n = 8)\), Tbx5fl+/R26CreERT2 \((n = 18)\), Gata4fl+/R26CreERT2 \((n = 12)\), and Gata4fl+/Tbx5fl+/R26CreERT2 \((n = 12)\) mice. Tbx5 heterozygotes displayed an irregular atrial electrogram, consistent with lack of P wave on surface ECG, which is representative of AF (F). A, atrial electrical signal; V, far-field ventricular electrical signal. (I) Pacing induction by intra-atrial pacing of mice in D-G. AF was reproducibly induced in 11 of 18 Tbx5 heterozygotes (60%) in contrast to 1 of 12 Gata4/Tbx5 compound heterozygotes, indicating rescue of atrial arrhythmias. \(P\) values were determined by Fisher’s exact test.
lar depolarization, was unchanged in Tbx5fl/+;R26CreERT2 compared with R26CreERT2 mice (P = 0.132) (Figure 1D and Supplemental Table 1), consistent with our previous publication (17). We further interrogated propensity to atrial arrhythmias by catheter-directed intracardiac pacing. Tbx5fl/+;R26CreERT2 adult mice were highly susceptible to AF induction, depicted by the irregular atrial electrogram, by intracardiac atrial pacing using either programmed single extra-stimulus or burst pacing. AF was reproducibly induced in 11 of 18 Tbx5fl/+;R26CreERT2 mice but in 0 of 8 R26CreERT2 mice following atrial burst pacing (P = 0.0074) (Figure 1, E, F, and I). Of these, 7 of 11 Tbx5fl/+;R26CreERT2 mice displayed atrial tachycardia (AT) and/or AF episodes lasting greater than 1,000 ms (P = 0.0128), with a mean duration of 15,963 ms. Remarkably, 5 of 11 Tbx5fl/+;R26CreERT2 mice displayed spontaneous atrial arrhythmias in addition to induced irregular atrial rhythm (Supplemental Figure 2). Tbx5fl/+;R26CreERT2 mice showed normal cardiac function 2 weeks after TM treatment, with no difference in left ventricular ejection fraction compared with R26CreERT2 adult mice (Supplemental Figure 3). These findings establish that Tbx5 haploinsufficiency causes atrial conduction deficits and increased AF risk.

Atrial rhythm was not sensitive to adult-specific Gata4 or Nkx2-5 haploinsufficiency. Adult-specific Gata4 (Gata4fl/+;R26CreERT2) or Nkx2-5 (Nkx2-5fl/+;R26CreERT2) haploinsufficiency caused no abnormalities of P-wave duration (P = 0.928 R26CreERT2 vs Gata4fl/+;R26CreERT2 and P = 0.08 R26CreERT2 vs Nkx2-5fl/+;R26CreERT2) or PR interval (P = 0.956 R26CreERT2 vs Gata4fl/+;R26CreERT2 and P = 0.985 R26CreERT2 vs Nkx2-5fl/+;R26CreERT2) by conscious ambulatory telemetry ECG and showed no sign of beat-to-beat variability by Poincaré analysis in comparison with R26CreERT2 control littermates (P = 0.946 R26CreERT2 vs Gata4fl/+;R26CreERT2 and P = 0.992 R26CreERT2 vs Nkx2-5fl/+;R26CreERT2) (Figure 1, C and D, Supplemental Figure 1, and Supplemental Table 1). Unlike reduced Tbx5 dosage, Gata4 or Nkx2-5 haploinsufficient mice were not vulnerable to atrial arrhythmias by pacing induction (Figure 1, F, G, and I, and Supplemental Figure 4). Specifically, 4 of 12 Gata4fl/+;R26CreERT2 mice (P = 0.116) and 1 of 8 Nkx2-5fl/+;R26CreERT2 mice (P = 0.5) experienced AF compared with 0 of 8 R26CreERT2 mice and both showed sta-
statistically less AF inducibility than Tbx5 haploinsufficiency \((P = 0.0074)\) (Figure 1, G and I, and Supplemental Figure 4B).

Remarkably, atrial arrhythmia caused by reduced Tbx5 dose was rescued by reduced Gata4 dose. Specifically, the increased \(P\)-wave duration observed in Tbx5-haploinsufficient mice was rescued in combined Gata4/Tbx5-haploinsufficient mice \((P = 0.0163)\) Gata4/Tbx5- compound heterozygote versus Tbx5\(^{+/-}\);R26\(^{CreERT2}\) (Figure 1C). Gata4/Tbx5-compound heterozygotes were not susceptible to AF induction by intracardiac burst pacing. Only 1 of 12 Gata4\(^{+/-}\);Tbx5\(^{+/-}\);R26\(^{CreERT2}\) mice reproducibly paced into AF, demonstrating rescue of AF inducibility compared with 11 of 18 for Tbx5\(^{+/-}\);R26\(^{CreERT2}\) mice \((P = 0.0068)\) and no greater propensity for AF induction compared with R26\(^{CreERT2}\) controls \((0 \text{ of } 8 \text{ R26}\(^{CreERT2}\), \(P = 0.999)\) (Figure 1, H and I).

Nkx2-5 haploinsufficiency had no discernable effect on atrial rhythm, by itself or in combination with Tbx5 or Gata4 haploinsufficiency. We analyzed adult-specific Tbx5/Nkx2-5 and Gata4/ Nkx2-5 compound heterozygotes and Gata4/Tbx5/Nkx2-5 triple heterozygotes for atrial conduction deficits or arrhythmia inducibility. No significant \(P\)-wave duration or PR interval differences were detected in any of these genotypes compared with their respective single- or double- haploinsufficient controls (Supplemental Figure 1, B and C, and Supplemental Table 1). Furthermore, no significant differences in atrial arrhythmia inducibility were observed in Gata4/Nkx2-5 \((P = 0.999)\) vs Gata4\(^{+/-}\);R26\(^{CreERT2}\) and \(P = 0.999\) vs Nkx2-5\(^{-/-}\);R26\(^{CreERT2}\) or Tbx5/Nkx2-5 \((P = 0.361\) vs Tbx5\(^{+/-}\);R26\(^{CreERT2}\) and \(P = 0.323\) vs Nkx2-5\(^{-/-}\);R26\(^{CreERT2}\)) compound heterozygotes compared with their respective single- heterozygote controls or in Gata4/Tbx5/Nkx2-5 triple heterozygotes \((P = 0.999)\) (Supplemental Figure 4). Specifically, 1 of 12 Gata4\(^{+/-}\);Nkx2-5\(^{-/-}\); R26\(^{CreERT2}\) mice paced into AF compared with control littermates, suggesting that GATA4 and Nkx2-5 do not interact together in the adult heart for control of atrial rhythm (Supplemental Figure 4, D and F). Tbx5\(^{+/-}\);Nkx2-5\(^{-/-}\);R26\(^{CreERT2}\) mice were susceptible to AF induction by intracardiac burst pacing, with prevalence identical to that of Tbx5 heterozygotes, indicating that Tbx5 and Nkx2-5 do not interact synergistically or antagonistically in the adult atrium (Supplemental Figure 4, C and F). Finally, mice lacking 1 copy of Gata4, Tbx5, and Nkx2-5 (Tbx5\(^{+/-}\);Gata4\(^{+/-}\);Nkx2-5\(^{-/-}\);R26\(^{CreERT2}\)) were not vulnerable to AF induction (Supplemental Figure 4, E and F). Thus, the AF susceptibility observed in Tbx5/Nkx2-5 compound heterozygotes was rescued by reducing Gata4 gene dosage, similar to Tbx5\(^{+/-}\);Gata4\(^{+/-}\);R26\(^{CreERT2}\) mice. In each genetic context, Nkx2-5 was dispensable, causing no worsening or improvement of atrial rhythm in adult mice compared with littermate controls.

Abnormal atrial electric activity observed in Tbx5 heterozygotes is normalized in Gata4/Tbx5 compound heterozygotes. We sought to define the cellular basis by which reduced Gata4 dose rescued the atrial conduction deficits and arrhythmia propensity caused by reduced Tbx5 dose. We previously showed that adult-specific Tbx5 haploinsufficiency caused prolonged atrial cardiomyocyte (CM) action potentials (APs) and abnormal spontaneous depolarizations of atrial myocytes, electrophysiological deficits that can cause or contribute to AF \((17)\). We hypothesized that Gata4 haploinsufficiency may rescue the cellular electrophysiology defects caused by Tbx5 haploinsufficiency. APs from atrial myocytes isolated from Tbx5\(^{+/-}\);R26\(^{CreERT2}\) mice 2 weeks after TM treatment were significantly prolonged, specifically in phases 2 and 3 of the AP and time to 90% repolarization (APD90), compared with R26\(^{CreERT2}\) atrial myocytes \((P = 0.0029)\) (Figure 2, A, B, and E, and Supplemental Table 2). EADs and DADs were frequently observed in Tbx5\(^{+/-}\); R26\(^{CreERT2}\) atrial myocytes but never in control littermates \((9 \text{ of } 14 \text{ Tbx5}\(^{+/-}\);R26\(^{CreERT2}\) vs 0 of 9 R26\(^{CreERT2}\) atrial myocytes, \(P = 0.003)\), consistent with our previous study (Figure 2, F-H, and ref. 17).

Adult-specific Gata4 haploinsufficiency caused no aberrations in AP measurements or inappropriate afterdepolarizations (Figure 2, C, E, and H, and Supplemental Table 2). Remarkably, decreased Gata4 dose rescued the cellular electrophysiology abnormalities observed in Tbx5\(^{+/-}\);R26\(^{CreERT2}\) mice. Atrial AP duration was rescued in Gata4\(^{+/-}\);Tbx5\(^{+/-}\);R26\(^{CreERT2}\) myocytes \((P = 0.0006)\) (Figure 2, B, D, and E). Additionally, propensity of EADs and DADs was rescued in Gata4\(^{+/-}\);Tbx5\(^{+/-}\);R26\(^{CreERT2}\) compared with Tbx5\(^{+/-}\); R26\(^{CreERT2}\) atrial myocytes \((P = 0.018)\), and Gata4\(^{+/-}\);Tbx5\(^{+/-}\); R26\(^{CreERT2}\) atrial myocytes showed no increased propensity for inappropriate depolarizations compared with R26\(^{CreERT2}\) control atrial myocytes \((P = 0.493)\) (Figure 2H). We conclude that the cellular electrophysiology defects caused by decreased Tbx5 dose were rescued by reduced Gata4 dose (Figure 2I).

Atrial myocyte ectopic activity, implicated as a mechanism of AF induction, can result from abnormal calcium handling. We therefore investigated altered expression of genes controlling cardiomyocyte calcium flux as a potential molecular mechanism for paroxysmal AF induction in Tbx5 heterozygote mice. We focused on calcium-handling genes involved in phase 2 and 3 of the AP, given our observation of specific deficits during these AP phases in Tbx5 adult haploinsufficient mice (Figure 2). Adult-specific Tbx5 heterozygote mice showed significantly diminished left atrial expression of Atp2a2 and Ryr2 compared with R26\(^{CreERT2}\) mice \((P = 0.0038 \text{ for } \text{Atp2a2}, P = 0.0163 \text{ for } \text{Ryr2})\), whereas expression of other calcium-handling genes, including Slc8a1, Slc8a6, and Cacna1c were not significantly altered (Figure 3A and Supplemental Table 3). Interestingly, Atp2a2 and Ryr2 expression was normalized in Gata4/Tbx5 compound heterozygotes \((P = 0.0232 \text{ for } \text{Atp2a2}; P = 0.0323 \text{ for } \text{Ryr2})\) (Figure 3A and Supplemental Table 3). In addition, we also assessed expression of potassium-handling genes previously linked to AF. RNA expression of Kcnj3 \((P = 0.0443)\), Kcnj5 \((P = 0.0039)\), and Kcnh2 \((P = 0.0182)\) showed significant reduction in left atrial tissue of Tbx5\(^{+/-}\);R26\(^{CreERT2}\) mice compared with control littermates (Supplemental Figure 5), whereas expression of Kcnas5, Kcnas3, Kcnk2, Kcnn3, and Kcnq1 were unchanged. However, Kcnj3, Kcnj5, or Kcnh2 gene expression was not normalized in Gata4\(^{+/-}\);Tbx5\(^{+/-}\); R26\(^{CreERT2}\) mice \((P = 0.7001 \text{ for } \text{Kcnj3}, P = 0.4254 \text{ for } \text{Kcnj5} \text{ and } P = 0.5286 \text{ for } \text{Kcnh2})\), respectively. Overall, these results suggested that atrial arrhythmogenesis in adult Tbx5 heterozygous mice may be mediated by altered sarcoplasmic reticulum (SR) calcium flux, mediated by reduced expression of Atp2a2 and Ryr2.

We previously demonstrated that TBX5 drives atrial expression of Pitx2 and that Tbx5 and PITX2 oppositely modulate the expression of cardiac rhythm effector genes, including Ryr2 and Atp2a2 \((17)\). This finding suggested that the rescue of Tbx5 haploinsufficiency by Gata4 haploinsufficiency could occur through diminished Pitx2 levels. Pitx2 mRNA expression remained unchanged.
in Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} and Gata4\textsuperscript{fl/+};R26\textsuperscript{CreERT2} mice 2 weeks after TM treatment, as previously described \((P = 0.7049, Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} compared with R26\textsuperscript{CreERT2} and \(P = 0.7261, Gata4\textsuperscript{fl/+};R26\textsuperscript{CreERT2} compared with R26\textsuperscript{CreERT2})\) (Figure 3B and ref. 17). Levels of Pitx2 mRNA were slightly higher in Gata4\textsuperscript{fl/+};Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} mice compared with WT littermates \((P = 0.0117); however, this increase was not significant compared with Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} or Gata4\textsuperscript{fl/+};R26\textsuperscript{CreERT2} mice \((P = 0.1208 vs Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} and \(P = 0.1135 vs Gata4\textsuperscript{fl/+};R26\textsuperscript{CreERT2} mice, respectively). This observation suggested that the rescue of Tbx5 haploinsufficiency by Gata4 haploinsufficiency was not mediated by a reduction of Pitx2 expression.

We hypothesized that Tbx5 and GATA4 directly coregulate expression of Atp2a2 and Ryr2. To test this hypothesis, we defined regions with overlapping chromatin occupancy for both TBX5 and GATA4 from published ChIP datasets (Figure 3, C and D, Supplemental Figures 6 and 7, and ref. 53). Candidate cis-regulatory elements (CREs) were refined by open chromatin regions from the HL-1 cardiomyocyte ATAC-seq dataset (54). We functionally interrogated these CREs for enhancer activity in the presence of TBX5 and/or GATA4. The Atp2a2 enhancer (mm9 Chr5: 122970476-122971591) demonstrated activation in response to TBX5 expression in human embryonic kidney (HEK) 293T cells, as previously described (Figure 3E and ref. 54). GATA4 similarly activated the enhancer and coexpression of TBX5, and GATA4 had no additive or synergistic effects by in vitro luciferase reporter assay in HEK293T cells (Figure 3E). In contrast, the Ryr2 enhancer was activated by TBX5 \((P = 0.0013)\) but not GATA4 alone \((P = 0.5923)\) in HEK293T cells (Figure 3F). Remarkably, co-expression
of GATA4 suppressed TBX5-dependent transactivation of this Ryr2 enhancer \( (P = 0.0681 \text{ vs } \text{TBX5 alone}) \), suggesting antagonistic interactions of TBX5 and GATA4 on this CRE. We further tested the Ryr2 CRE in HL-1 cardiomyocytes, which possesses expression of cardiac transcription factors, including TBX5 and GATA4 \( (55) \). This enhancer previously demonstrated TBX5-dependent activity in HL-1 cells, and consistently, this enhancer alone showed activation in HL-1 cardiomyocytes \( (54) \). Interestingly, mutation of the 4 GATA binding motifs within this enhancer caused an increase of enhancer activity in HL-1 cardiomyocytes \( (P = 0.0010 \text{ vs } \text{WT enhancer}) \) (Figure 3G). These observations indicated that GATA4 represses TBX5-dependent activity of the Ryr2 enhancer.

**Reduced SR load and SERCA function caused by Tbx5 haploinsufficiency is rescued by Gata4 haploinsufficiency.** Abnormal CM membrane depolarizations, including DADs, can be induced by Ca\(^{2+}\)-driven Na\(^+/\)Ca\(^{2+}\) exchanger (NCX) activity in the setting of reduced SERCA-mediated SR Ca\(^{2+}\) uptake, causing membrane depolarization \( (56) \). We therefore hypothesized that the ectopic depolarizations caused by Tbx5 haploinsufficiency would be associated with depressed SERCA function and slowed SR Ca\(^{2+}\) uptake. To test this hypothesis, we measured SR load and SERCA activity in Tbx5 heterozygote atrial myocytes, 2 weeks after TM treatment. We used nonfluorescent acetoxymethyl ester (Fluo-4 AM) to study cytosolic calcium-handling kinetics and Fura-2 acetoxymethyl ester (Fura-2 AM) to study diastolic calcium levels. We defined SR Ca\(^{2+}\) uptake by loading myocytes with Fluo-4 AM and pacing with a train-of-field stimuli to achieve a steady-state load followed by application of caffeine to synchronize ryanodine receptor opening, resulting in a maximum release of SR calcium into the cytosol (Figure 4, A–D). We observed reduced caffeine-induced calcium-transient amplitudes in Tbx5\(^{+/+}\);R26\(^{CreERT2}\) compared with R26\(^{CreERT2}\) mice, indicating reduced SR calcium levels \( (P < 0.0001) \) (Figure 4, A, B, and F). To assess SERCA activity, we measured [Ca\(^{2+}\)]\(_{\text{cyt}}\) decay rate after caffeine in the absence of external Nao in TM-treated and rescued by Gata4 haploinsufficiency. P values were determined by 1-way ANOVA followed by post-hoc Tukey test. (F) SERCA activity determined from the maximal rate of calcium decay was diminished in Tbx5\(^{+/+}\);R26\(^{CreERT2}\) compared with R26\(^{CreERT2}\) and normalized by Gata4 haploinsufficiency. P values were determined by 1-way ANOVA followed by post-hoc Tukey test. (G) Calcium homeostasis in control atrial myocytes is disrupted by Tbx5 haploinsufficiency and rescued by decreasing Gata4 gene dosage.
Figure 5. Normalization of SERCA2 function eliminates AF susceptibility in Tbx5fl/++;R26CreERT2. (A–D) Representative SERCA2 traces of R26CreERT2 (A), Tbx5fl/++;R26CreERT2 (B), Pln+/−;R26CreERT2 (C), and Tbx5fl/++;Pln+/−;R26CreERT2 (D) atrial myocytes. (E and F) Representative SR load and SERCA2 measurements from A–D. SR load (E) and SERCA2 function (F) was low in Tbx5fl/++;R26CreERT2 and normalized in Tbx5/Pln compound heterozygous mice (n = 43 R26CreERT2, n = 40 Tbx5fl/++;R26CreERT2, n = 30 Pln+/−;R26CreERT2, and n = 18 Tbx5fl/++;Pln+/−;R26CreERT2). For SR load and SERCA2 measurements, 3–4 mice per genotype were analyzed. (G–J) Intracardiac atrial electrogram recordings and corresponding surface ECG of R26CreERT2 (n = 9), Tbx5fl/++;R26CreERT2 (n = 6), Pln+/−;R26CreERT2 (n = 8), and Pln+/−;Tbx5fl/++;R26CreERT2 (n = 7) mice. Tbx5 heterozygotes displayed irregular atrial electrogram, representative of AF (H). A, atrial electrical signal; V, far-field ventricular electrical signal. (K) Pacing induction by intra-atrial pacing of mice in G–J. AF was reproducibly induced in 4 of 6 Tbx5 heterozygotes (60%) in contrast to 0 of 7 Pln/Tbx5 compound heterozygotes, indicating complete rescue of atrial arrhythmias. P values were determined by Fisher’s exact test; *P < 0.05. (L) Calcium homeostasis in control atrial myocytes is disrupted by Tbx5 haploinsufficiency and rescued by decreasing Pln gene dosage.
**Figure 6. TBX5 and GATA4 are key regulators of atrial calcium homeostasis.** Proteins involved in Ca\(^{2+}\) handling of atrial myocytes. In the healthy atrium, calcium homeostasis is maintained through a tight balance between Ca\(^{2+}\) fluxes across the membrane. Reducing Tbx5 gene dosage in the adult heart results in depressed Atp2a2 expression and SERCA2 function, affecting SR Ca\(^{2+}\) influx. Abnormal Ca\(^{2+}\) handling leads to Ca\(^{2+}\) accumulation in the cytosol, contributing to ectopic atrial activity, prolonged APs, and atrial arrhythmias observed with adult-specific Tbx5 haploinsufficiency. Reducing Gata4 gene dosage rescues Atp2a2 expression, SERCA2 function and SR Ca\(^{2+}\) uptake, restoring sinus rhythm. SERCA2 and its inhibitory protein PLN play a fundamental role in Ca\(^{2+}\) handling within atrial myocytes. Reducing Pln gene dosage, as a means to modulate SERCA2 function, normalizes SERCA2 activity and SR Ca\(^{2+}\) uptake, resulting in restoration of sinus rhythm in combined Tbx5/Pln haploinsufficiency.

from R26\textsuperscript{CreERT2} control cardiomyocytes (P = 0.312), and Gata4\textsuperscript{fl/+}; Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} myocytes showed a significantly increased SR load compared with Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} cardiomyocytes (P = 0.0070) (Figure 4, B, D, and E). Furthermore, SERCA activity was normalized in Gata4\textsuperscript{fl/+};Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} myocytes as observed by a normal decay rate upon removal of caffeine, compared with Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} cardiomyocytes (P = 0.0145) (Figure 4, B, D, and F). Additional analysis of the calcium dependence of SERCA activity showed decreased activity at all levels of cytosolic calcium in Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} compared with R26\textsuperscript{CreERT2}, and this was normalized in Gata4\textsuperscript{fl/+};Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} mice (Supplemental Figure 8). We conclude that the atrial calcium homeostasis defects caused by reduced Tbx5 dose were rescued by reduced Gata4 dose, providing a molecular mechanism for the physiological rescue (Figure 4G).

**Modulation of SERCA function eliminates TF-driven arrhythmogenic phenotype.** The observation that reduced Gata4 dose rescued the defects caused by a reduced Tbx5 dose at the level of cardiac rhythm control, cellular electrophysiology, gene expression, and reduced SR calcium flux suggested that the pathophysiology of reduced Tbx5 dose on atrial rhythm control may be entirely mediated by reduced SR calcium uptake.

PLN inhibits SERCA activity in its nonphosphorylated state. Pln is essential for atrial calcium homeostasis, and human genetic variants at PLN associate with AF risk, suggesting that PLN activity contributes to AF susceptibility (3, 57). We hypothesized that reducing Pln gene levels may restore SERCA activity and rescue atrial calcium abnormalities caused by reduced Tbx5 dose. We compared SR uptake, SERCA function, and AF inducibility in adult-specific littermate Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} mice 2 weeks after TM treatment. We observed depressed SR load (P < 0.0001) and SERCA activity (P = 0.0221) in atrial cardiomyocytes from Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} mice compared with those from R26\textsuperscript{CreERT2} littermate controls, as expected (Figure 5, A, B, E, and F). Pln heterozygote atrial cardiomyocytes showed a significant increase in SR load (P = 0.0304) and SERCA function (P = 0.0003) compared with those from R26\textsuperscript{CreERT2} (Figure 5, A, C, E, and F). Both SR load (P = 0.0211) and SERCA activity (P = 0.0007) were normalized in Tbx5/Pln compound heterozygotes (Figure 5L).

We hypothesized that normalization of SERCA function and SR load in Tbx5/Pln compound heterozygotes may rescue the AF predisposition caused by decreased Tbx5 dose. Tbx5 heterozygotes were highly susceptible to atrial arrhythmias, consistent with our previous observations (Figure 1, E and H). AF was induced in 4 of 6 Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2} mice compared with 0 of 10 for R26\textsuperscript{CreERT2} controls (P = 0.008) (Figure 5, H and K). Pln heterozygotes demonstrated no AF inducibility (0 of 8 mice; P = 0.999) (Figure 5, I and K). Remarkably, Tbx5\textsuperscript{fl/+};Pln\textsuperscript{+/–};R26\textsuperscript{CreERT2} adult mice were not susceptible to AF induction by intracardiac pacing (0 of 7 Tbx5\textsuperscript{fl/+};Pln\textsuperscript{+/–};R26\textsuperscript{CreERT2} mice; P = 0.021 vs Tbx5\textsuperscript{fl/+};R26\textsuperscript{CreERT2}) (Figure 5, G–K). Overall, we conclude that decreased Pln gene dose...
normalizes SERCA activity and rescues AF susceptibility caused by Tbx5 haploinsufficiency, indicating that diminished SERCA activity is a primary deficit causing arrhythmogenesis in the setting of Tbx5 haploinsufficiency (Figure 5L). TBX5 therefore regulates SERCA in both the atria and ventricles for both cardiac function and rhythm. The observation that decreased Pln normalizes SR Ca\textsuperscript{++} uptake and SERCA activity in a mouse model of AF provides a direct molecular link between calcium flux perturbations observed in HF and AF risk, which are strongly associated, regardless of genetic background. These observations suggest that PLN inhibition and more generally, the modulation of SR calcium flux, may be considered as possible therapeutic approaches for the treatment of AF in patients with HF.

Understanding of the genetic basis of cardiac rhythm control has benefited greatly from highly powered genome-wide association studies, which have identified more than 100 loci contributing to the heritability of AF. A mechanistic and actionable understanding of how the identified loci affect cardiac rhythm control requires the transition from genetic implication to functional investigation (71). The implication of the cardiogenic TFs TBX5, GATA4, and NKX2-5 by GWAS is an exciting feature, suggesting a shared transcriptional kernel in cardiac development and adult cardiac rhythm control. In the context of cardiac development, Tbx5, Gata4, and Nkx2-5 interact genetically in a synergistic fashion, their encoded TFs physically associate, and they cooperatively drive embryonic cardiac gene expression (33–44). These detailed studies provided a clear paradigm for the cooperative interaction between these TFs in adult rhythm control. Remarkably, we find that these TFs interact very differently in the adult mouse: Tbx5 and Gata4 act oppositely, and Nkx2-5 has no appreciable impact.

Understanding how Tbx5 and Gata4 levels are integrated at the molecular level to afford genetic rescue of the Tbx5 mutant phenotype by reduced Gata4 activity will be essential for AF risk prediction. We identified an enhancer at Ryr2 that molecularly integrates GATA4 and Tbx5 with opposite activity. Previous work has characterized multiple promoters or enhancers coregulated by Tbx5 and Gata4, and in each case, positive interactions were observed, either additive or synergistic (33, 36–41). In a few cases, however, GATA4 has been reported to possess repressive molecular activity on cardiac enhancers. For example, GATA4 recruits Hdac1/2 to deacetylate specific atrioventricular canal loci, thereby repressing AV canal identity during cardiac chamber development (57). GATA4 can also cooperate with -catenin on TCFL2-enhancers in the adult heart, to maintain normal homeostasis through repression of TCFL2-driven loci (72). Therefore, GATA4 harbors repressive potential in some contexts in the heart. Here, we identified a repressive role for GATA4 at an enhancer of Ryr2, on which GATA4 opposes Tbx5-dependent activation (Figure 3). We observed that Tbx5 activation and GATA4 antagonism of the Ryr2 enhancer was dependent on GATA-binding sites (73). However, we observed that GATA4 antagonized Tbx5-dependent activation (Figure 3). We observed that Tbx5 activation and GATA4 antagonism of the Ryr2 enhancer was dependent on GATA-binding sites in HL-1 cells but not HEK293T cells. One possibility for these different results is the distinct physiological conditions of these cell lines. Because HEK cells lack endogenous expression of Tbx5 and GATA4, TF overexpression may overcome the effect of the binding-site mutation because Tbx5 and GATA4 physically interact (33). In contrast, HL-1 cells, possessing endogenous physiologic expression of the cardiogenic kernel of TFs, including Tbx5 and GATA4, may be more sensitive to the necessity of the GATA binding sites. It remains to be elucidated how GATA4 antagonizes Tbx5-dependent function in the adult atrium, perhaps by recruiting repressive chromatin-remodeling enzymes or cardiac corepressors. Nonethe-
less, our observations provide a model for the integration of TBX5 and GATA4 dose on gene expression by the opposite modulation of single-target gene enhancers. Understanding the molecular mechanisms underlying the opposite action of TBX5 and GATA4 on cardiac gene expression will improve our understanding of the molecular basis of adult cardiac rhythm control.

This work unveiled complex genetic interactions between genes individually implicated in AF by human genetic studies. The opposite sign with which decreased Gata4 dose affected AF risk in the context of decreased Tbx5 dose indicates the importance of unveiling specific genetic interactions between genetic risk loci for understanding the combined impact of genetic variants across genomes for disease risk prediction. Human genetic studies have been underpowered for the identification of multigenic interactions to date, highlighting the importance of gene-gene interaction studies in model systems such as those included in this study. This work suggests that interaction studies between genetic risk loci will be an essential component of understanding personalized risk from genetic association studies.

Methods

Experimental design. This study was designed to investigate the oligogenic interaction of TFs that have been linked to AF by GWAS studies. We used murine models of conditional Tbx5 deletion, conditional Gata4 deletion, conditional Nkx2-5 deletion, and germline Pln heterozygosity for their similarities to human AF phenotypes. The number of mice per genotype depended on the experiments and is specified in figure legends. For animal studies, littermates were used as controls, and mice were grouped when appropriate. Endpoints for studies were selected according the phenotype of adult Tbx5-haploinsufficient mice. For single-cell electrophysiology and calcium flux measurements, 3–5 mice per genotype were analyzed, and a total of 10 cells were recorded for every mouse. All experiments, recordings, and analysis were performed in a blinded fashion. Outliers were excluded if samples/replicates were greater than 2 SDs from the population mean.

Transgenic mice. Tbx5\textsuperscript{fl/fl}, Gata4\textsuperscript{fl/fl}, Nkx2-5\textsuperscript{fl/fl}, Pln\textsuperscript{+/−}, and Rosa26\textsuperscript{CreERT2} mice have all been previously described (28, 43, 52, 73, 74). Mice were maintained on a mixed genetic background, harboring 1 copy of the Cre recombinase. All experiments involving Tbx5, Gata4, Nkx2-5, and Pln heterozygotes and Gata4\textsuperscript{−/−}, Tbx5\textsuperscript{−/−}, Nkx2-5\textsuperscript{−/−}, and Tbx5/Pln compound heterozygotes and Tbx5/Gata4\textsuperscript{−/−}, Nkx2-5\textsuperscript{−/−} triple heterozygotes were performed 2 weeks after TM treatment, and age-matched littermate controls (R26\textsuperscript{CreERT2}) were used for comparison. A total of 200 μl of tamoxifen (20 mg/ml in corn oil) was administered by i.p. injection for 3 consecutive days at 6 to 8 weeks of age, as previously described (17). Mice were anesthetized with isoflurane, and right atrial and ventricular electrograms as well as surface electrograms were recorded using a 1.1-F octopolar catheter (EPR-800, Millar Instruments) inserted via the right jugular vein. Programmed extra-stimulation protocols and burst pacing were used to induce AT and AF in animal subjects. Programmed right atrial extra stimulation was carried out using S1 drive trains of 80 ms followed by 5 extra stimulations at 50 ms each. Burst pacing was performed by applying a series of single extra stimulus delivered at a constant pacing rate of 15–20 ppm (900–1200 bpm). Inducibility was considered positive if 2 or more series of AT and/or fibrillation was observed following burst pacing in the same animal. Fibrillation was considered noninducible if 0 or 1 cycle or irregular atrial rhythm was observed in the animal.

Echocardiography. Transthoracic echocardiography was performed 2 weeks after TM treatment under inhaled isoflurane, delivered via a nose cone, as previously described (76). Briefly, animals were imaged with a VisualSonics Vevo 770 machine (VisualSonics) using a 30-MHz high-frequency transducer. Body temperature was maintained using a heated imaging platform. Two-dimensional images were recorded in parasternal long- and short-axis projections, with guided M-mode recordings at the midventricular level in both views. M-mode echocardiographic images were obtained at the midpapillary muscle level in the parasternal short-axis view and measurement calculated at the same level from M- and/or B-mode images of long- or short-axis view. Left ventricular dimensions in both diastole and systole were measured from at least 3 different beats from each projection and averaged to calculate the left ventricular ejection fraction.

Calcium flux measurement. Single-cell atrial cardiomyocytes were isolated by Langendorff perfusion with 2 mg/ml of Collagenase Type 2 at 5 ml/min. Atrial myocytes were plated on laminin-coated glass-bottom dishes and incubated at room temperature for 30 minutes prior to incubation with Fluo-4 AM (Molecular Probes/Invitrogen). Cells were then incubated with 10-μM Fluo-4 AM for 1 hour in normal Tyrode’s solution containing (in mM): 140 NaCl, 4 KCl, 10 glucose, 10 HEPES, and 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, with pH 7.4 using NaOH, followed by a 10-minute perfusion wash with pre-warmed Tyrode’s solution. SERCA and NCX measurements were performed using an Olympus microscope with a x20 objective lens, a LAMBDA DG-4 power source with 488-nm excitation and 515-nm emission filters and a PMT (Microphotometer) to record whole-cell signal, with electrical field stimulation (Grass stimulus; Astro-Med) at 1 Hz. SERCA and NCX activities were measured as follows: 10-mM caffeine-containing solution was applied in the absence of extracellular sodium and the cells returned to normal Tyrode’s solution at the end of the recording. In the presence of sodium-free caffeine Tyrode’s solution, the intracellular calcium remains elevated, and the peak value is a measure of SR calcium load that was released into the cytosol.

Whole-cell electrophysiological recordings. Action potentials were recorded using the whole-cell patch-clamp method (17). Whole-cell action potentials were recorded using an Axopatch-200B amplifier connected to a Digidata1550A acquisition system (Axon Instruments). Atrial myocytes were isolated by Langendorff perfusion, plated on laminin-coated glass-bottom dishes and incubated at room temperature for 30 minutes before recordings. Tyrode’s solution (140-mM NaCl, 4-mM KCl, 1-mM MgCl\textsubscript{2}, 1-mM CaCl\textsubscript{2}, 10-mM HEPES, and 10-mM glucose, pH 7.4 with NaOH) was used to perfuse atrial cardiomyocytes during recordings at 37°C. Internal pipette solution contained 20-mM
KCl, 100-mM K-glutamate, 10-mM HEPES, 5-mM MgCl₂, 10-mM NaCl, 5-mM Mg-ATP, and 0.3-mM Na-GTP. Action potentials were triggered using 0.5 nA x 2-ms current clamp pulses following liquid junction potential correction. All recordings were filtered at a frequency of 2 kHz using a built-in Bessel filter and sampled at 10 kHz. Results were analyzed using pCLAMP10 (Axon Instruments).

**Diastolic calcium measurements.** Atrial myocytes were isolated by Langendorff perfusion, plated on laminin-coated glass-bottom dishes, and incubated at room temperature for 30 minutes before staining with Fura-2 AM for 10 minutes in normal Tyrode’s solution. Diastolic calcium measurements were performed at a fluorescence emission of 510 nm and recorded using an Olympus IX81 Inverted Widefield Microscope. [Ca]i was calculated by the ratio of emissions following excitation at 340 nm and 380 nm.

**Relative luciferase assays.** HEK293T and HL-1 cells were cotransfected as described previously (17). The Atp2a2 and Ry2 cis-regulatory elements were amplified from C57/B6 mouse genomic DNA. The sequence was confirmed by sequencing and then cloned into the pGL4.23 enhancer luciferase response vector with a minimal promoter. Mutagenesis of the GATA4-binding motifs was performed by PCR cloning, confirmed by sequencing and then cloned into the pGL4.23 vector. Cells were co-transfected with the luciferase response vector and pRL control using lipofectamine 3000, then lysed and assayed following 48 hours using the Dual-Luciferase Reporter Assay system (Promega).

**Quantitative real-time PCR.** Total RNA was extracted from the left atrial wall of 8- to 10-week-old adult mice (2 weeks after receiving TM) using Trizol (Invitrogen) combined with RNeasy mini-kit (Qiagen), according to the manufacturer’s instructions. First-strand cDNA was synthesized using the qScript cDNA synthesis kit (Quanta) according to the manufacturer’s protocol. Gene expression was assayed using the Power SYBR Green PCR Master Mix (Applied Biosystems) and run on an Applied Biosystems AB7500 machine in 96-well plates. Relative fold changes were calculated using the comparative threshold cycle method (2-ΔΔCt) using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as an internal control. Primer sequences used in this study are listed in Supplemental Table 3.

**Statistics.** All data are represented as means ± SEM. For comparison of conscious ECG parameters, action potential duration (APD50 and APD90), SR load, and SERCA activity, a 1-way ANOVA followed by post-hoc Tukey analysis was used to test significance. For gene-expression studies and in vitro luciferase assays, a 1-way ANOVA followed by internal Student’s t test was used to test significance. AF inducibility and triggered activity (from AP measurements) counts were analyzed with a Fisher’s exact test (2-tailed).

**Study approval.** All animal experiments were performed in accordance with national and institutional guidelines and were approved by the University of Chicago Institutional Animal Care and Use Committee.

**Author contributions**

BL and WD were involved in experimental design, execution, and analysis, wrote the manuscript, and performed statistical analysis. BL performed and analyzed ambulatory ECG, whole-animal electrophysiology studies, and gene expression. WD performed and analyzed calcium flux measurements. LT performed and analyzed single-cell electrophysiology experiments. SL, KMS, and MG performed and analyzed luciferase assays. MTB performed and analyzed intracardiac electrophysiology studies. CRW performed and analyzed single-cell electrophysiology experiments and wrote the manuscript. IPM was involved in the design, execution, and analysis of experiments and wrote the manuscript.

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