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Circulation Research

ORIGINAL RESEARCH

Identification of Functional Variant Enhancers Associated With Atrial Fibrillation

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RATIONALE: Genome-wide association studies have identified a large number of common variants (single-nucleotide polymorphisms) associated with atrial fibrillation (AF). These variants are located mainly in noncoding regions of the genome and likely include variants that modulate the function of transcriptional regulatory elements (REs) such as enhancers. However, the actual REs modulated by variants and the target genes of such REs remain to be identified. Thus, the biological mechanisms by which genetic variation promotes AF has thus far remained largely unexplored.

OBJECTIVE: To identify REs in genome-wide association study loci that are influenced by AF-associated variants.

METHODS AND RESULTS: We screened 2.45 Mbp of human genomic DNA containing 12 strongly AF-associated loci for RE activity using self-transcribing active regulatory region sequencing and a recently generated monoclonal line of conditionally immortalized rat atrial myocytes. We identified 444 potential REs, 55 of which contain AF-associated variants ($P<10^{-8}$). Subsequently, using an adaptation of the self-transcribing active regulatory region sequencing approach, we identified 24 variant REs with allele-specific regulatory activity. By mining available chromatin conformation data, the possible target genes of these REs were mapped. To define the physiological function and target genes of such REs, we deleted the orthologue of an RE containing noncoding variants in the *Hcn4* (potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4) locus of the mouse genome. Mice heterozygous for the RE deletion showed bradycardia, sinus node dysfunction, and selective loss of *Hcn4* expression.

CONCLUSIONS: We have identified REs at multiple genetic loci for AF and found that loss of an RE at the *HCN4* locus results in sinus node dysfunction and reduced gene expression. Our approach can be broadly applied to facilitate the identification of human disease-relevant REs and target genes at cardiovascular genome-wide association studies loci.

GRAPHICAL ABSTRACT: A graphical abstract is available for this article.

Key Words: atrial fibrillation ■ chromatin ■ gene expression ■ genetics ■ genome-wide association study ■ regulation ■ STARR-seq ■ variants

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Atrial fibrillation (AF) is the most common cardiac arrhythmia, with a prevalence increasing with age. It is a major risk factor for embolic stroke and is associated with morbidity and mortality.[1] Although factors such as sex, aging, and comorbidities contribute to AF risk, family studies have shown there is a heritable component to AF.[2–4] With the advance of genome-wide association studies (GWAS), the search for the genetic components of AF has accelerated. Many single-nucleotide polymorphisms (SNPs) were found to be associated with AF in over 100 different loci, where each of the lead SNPs at a locus is often in strong linkage disequilibrium with hundreds of other common variants with virtually indistinguishable statistical associations.[5–12] The vast majority of these AF-associated SNPs are found in intergenic/intronic regions rather than protein-coding regions, which when coupled with complex linkage disequilibrium structure makes identifying causal variant(s) challenging. This topic is a priority of the field, as identifying causal variant(s) at GWAS loci is the first step in

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Novelty and Significance

What Is Known?

- Genome-wide association studies have identified over 100 mostly noncoding regions of the genome that contain genetic variants (single-nucleotide polymorphisms) and variants in linkage disequilibrium associated with atrial fibrillation (AF).
- Among the many variants, many are likely just in the vicinity of AF-associated genetic elements, but others may directly affect AF susceptibility by, for instance, altering the activity of transcriptional regulatory elements and potentially the expression of effector genes, thus leading to increased AF risk.
- The challenge of genome-wide association study interpretation lies in the identification of the causative variants, the directionality of altered target gene expression, and the effect on biological mechanisms that impact on the trait of interest such as AF.

What New Information Does This Article Contribute?

- We systematically screened 12 loci strongly associated with AF for atrial regulatory element activity using a combination of self-transcribing active regulatory region sequencing and a conditionally immortalized rat atrial cardiomyocyte line.
- We identified over 400 potential human regulatory elements and validated one such element in a murine in vivo model using genome editing, showing that its deletion causes decreased *Hcn4* expression and bradycardia.
- This specific approach may be broadly applicable to interpretation of genome-wide association study data by aiding in the dissection of biological mechanisms underlying disease predisposition, stratification of patients based on genotype, and potentially leading to the development of personalized diagnostics or therapies.

AF is the most common cardiac arrhythmia and is associated with significant morbidity and mortality. Genome-wide association studies have identified over 100 mostly noncoding regions in the genome containing common variants associated with AF. Although poorly understood mechanistically, such variants could influence the function of critical gene regulatory elements, thus linking them to AF risk. Using an inducible atrial cardiomyocyte cell line, we performed a high-throughput functional assay on 12 highly associated variant regions, totaling over 2 million base pairs. We identified over 400 potential human regulatory elements in the AF-associated regions and identified 24 variants that cause altered activity of such elements. The homologue of a noncoding variant region close to *Hcn4* was deleted from the mouse genome by genome editing to assess its function in vivo. These results provide a first step in the functional dissection of the AF-associated regions and could help pave the way to further stratify patients based on genotype.

Nonstandard Abbreviations and Acronyms

AF	atrial fibrillation
BAC	bacterial artificial chromosome
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9
DEL	deletion
ESRRB	estrogen-related receptor-beta
GWAS	genome-wide association study
iAM	inducible atrial cardiomyocyte
IRF	interferon regulatory factor
L2FC	log2 fold change
MEF2	myocyte enhancer factor 2
RE	regulatory element
sgRNA	guide RNA
SNP	single-nucleotide polymorphism
STARR-seq	self-transcribing active regulatory region sequencing
TF	transcription factor

determining the molecular mechanism(s) by which these variants increase the risk of AF.

It has been found that disease-associated variants are often located in regions that are enriched for epigenetic signatures such as DNA accessibility and protein-binding sites, which predict the presence of *cis*-regulatory elements (REs).[13–15] This suggests that alterations in target gene expression are due to disease-associated variants that modify the function of REs, by, for example, disruption or creation of TF (transcription factor)-binding sites.[15,16] This likely causes small but important differences in the amount of RNA transcribed and protein produced, thereby predisposing to AF development.[17] Because AF is a complex disease, it is thought that multiple variants from different loci may act in an additive way to environmental risk factors to cause disease. Expression quantitative trait locus studies linking AF-associated variants to changed expression in heart tissue support this notion, as effect sizes on gene expression are often small.[18]

To determine which AF-associated SNPs cause an effect, an unbiased approach to identify variants that alter regulatory DNA sequences involved in the regulation of atrial gene expression is needed. To this end, we screened 12 loci strongly associated with AF spanning 2.45 Mbp

of human genomic sequence using the high-throughput method self-transcribing active regulatory region sequencing (STARR-seq)[19] in a recently generated line of fully differentiation-competent rat atrial myocytes designated inducible atrial cardiomyocyte (iAM)-1.[20] Of the genetic regions showing RE activity in this assay, we selected those regions that overlap both the locations of AF-associated variants and other epigenetic signatures suggestive of regulatory activity for further analysis. Subsequently, we used an adaptation of the STARR-seq approach to determine which of the selected AF-associated variants confer allele-specific differential enhancer activity. Moreover, we suggest potential target genes of these allele-specific REs by incorporation of promoter capture Hi-C data.[21] Furthermore, we deleted the mouse orthologue of a variant region with REs containing a variant that shows allele-specific activity in the *HCN4* locus, revealing its regulatory activity, as well as its target gene specificity in vivo. Our approach should prove useful for wider application to GWAS follow-up studies, aiding in the identification of regions with regulatory activity, as well as disease-associated functional variants.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. Detailed Methods are provided in the Data Supplement. For research materials listed in the Methods and Materials, please see the Major Resources Table in the Data Supplement.

Human Material

DNA was obtained from AF cases previously enrolled in the Massachusetts General Hospital AF Study.[18] All participants were of European ancestry, and the study is reviewed annually by the Institutional Review Board of the Massachusetts General Hospital.

Generation of Transgenic Mice Using CRISPR

Guide RNA (sgRNA) constructs were designed using the online tool ZiFiT Targeter.[22] For sgRNA sequences, see Table I in the Data Supplement. Cas9 and sgRNA constructs were transcribed in vitro with the MEGAshortscript T7 (Thermo Fisher Scientific; AM1354) and mMESSAGE mMACHINE T7 Transcription (Thermo Fisher Scientific; AM1344M). sgRNAs (10 ng/μL per sgRNA) and Cas9 mRNA (25 ng/μL) were injected into the cytoplasm of 1-cell FVB/NRj zygotes to generate founder mouse lines. Lines were maintained on an FVB/NRj background (Janvier). Animal care and experiments were performed in accordance with the national and institutional guidelines. In all experiments, both male and female animals were used.

RESULTS

STARR-seq Identifies Atrial Myocyte-Specific Regulatory Potential in AF-Associated Regions

To identify biologically relevant regulatory potential through high-throughput assays such as STARR-seq, the use of cell types that represent the tissue of interest and that can be efficiently transfected is critical. Therefore, we chose to use conditionally immortalized rat atrial myocytes (iAM-1 cells).[20] iAM-1 cells proliferate with a doubling time of 38 hours in the presence of doxycycline and spontaneously reacquire a phenotype closely resembling that of primary rat atrial myocytes when cultured in the absence of doxycycline. We hence anticipated that regulatory sequences normally active in the atria would be activated in these cells in the absence of doxycycline. We first determined that at day 9 of differentiation, iAM-1 cells are well differentiated and do not further mature (see extended Results in the Data Supplement; Figure I in the Data Supplement). Furthermore, reporter gene expression driven by human *NPPA* and *GJA1* promoter increased between days 0 and 9 of differentiation and stabilized (*NPPA*) or decreased (*GJA1*) afterward (Figure II in the Data Supplement). After optimization, a transfection efficiency of ≈50% of the iAM-1 cells was reached (Figure IIIA through IIID in the Data Supplement). Importantly, transfected cells differentiated equally well into functional (ie, excitable and contractile) cardiomyocytes as their nontransfected counterparts as judged from the results of bright-field microscopy and optical voltage mapping of the iAM-1 cultures at 9 days of differentiation (Figure IIIE through IIIH in the Data Supplement). We concluded that promoters of human cardiac genes are activated in redifferentiated iAM-1 cells and that day 9 is an optimal time point for STARR-seq analysis.

We next used STARR-seq[19] to determine the regulatory potential of a library of genomic fragments from regions containing AF GWAS SNPs (Figure 1A). We selected the 12 most significantly AF-associated GWAS loci (Table II in the Data Supplement) from a recent comprehensive AF GWAS.[11] This GWAS contained over 65 000 AF patients composed of European (84.2%), Japanese (12.5%), African American (2%), and Brazilian and Hispanic (1.3%) populations. Using a threshold of 5×10^{-8} and Bonferroni correction, 104 AF-associated loci were found, each containing a sentinel SNP, and a number of associated variants in linkage disequilibrium with the sentinel SNP. Fifteen human bacterial artificial chromosomes (BACs) were selected that cover the majority of the regions containing AF-associated sentinel and associated variants in linkage disequilibrium. In total, the BACs in this study span 2.45 Mbp, with an average size of 163 kbp. Together, they contain 1641 AF-associated variants at $P<10^{-8}$ and 2465 AF-associated variants at $P<10^{-4}$,[11] with an average of 113 variants per BAC (Table II in the Data Supplement). Because of the random fragmentation of the BACs, each genetic locus is represented in multiple fragments of different lengths in a continuous distribution over that locus, resulting in a higher resolution than is achieved by other techniques using discrete preselected regions.[23] A library

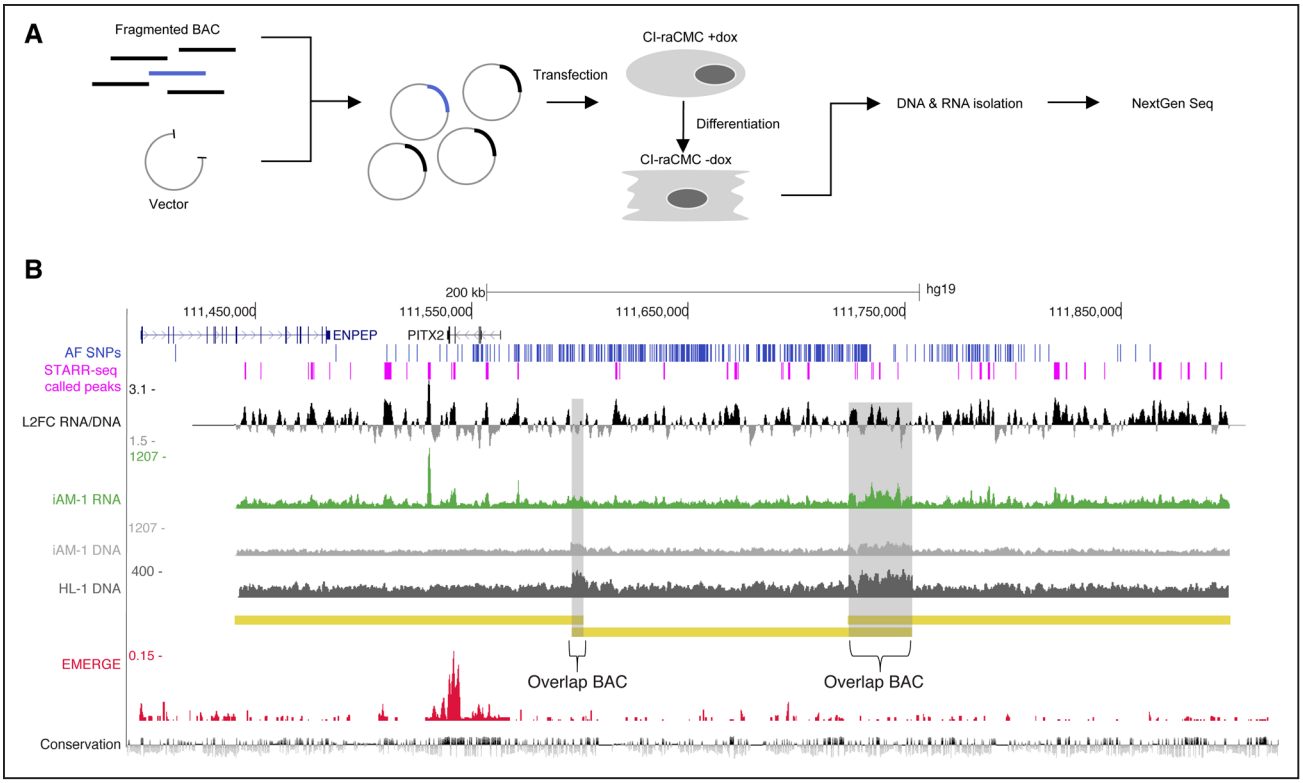

Figure 1. Self-transcribing active regulatory region sequencing (STARR-seq) approach and PITX2 locus output showing increased number of reads at overlapping bacterial artificial chromosomes (BACs).
A, Graphical representation of the STARR-seq approach. **B**, Example of *PITX2* (paired-like homeodomain 2) locus. Showing genome-wide association study single-nucleotide polymorphisms, called STARR-seq peaks, RNA sequencing (seq) and DNA-seq reads of inducible atrial cardiomyocyte (iAM)-1 cells, DNA-seq reads of cardiac cell line HL-1 cells (Methods in the Data Supplement), BAC locations, EMERGE output, and Genomic Evolutionary Rate Profiling score (GERP). Highlighted areas indicate BAC overlap, reflected by an increased presence of these regions in the DNA-seq data. dox indicates doxycycline; CI-raCMC indicates conditionally immortalized rat atrial myocytes; and L2FC, log 2 fold change.

was generated by inserting the fragments downstream of the SCP1 (super core promoter) promoter of plasmid (p)STARR-seq.[19]

The STARR-seq library was transfected before differentiation and after 9 days of redifferentiation; mRNA from 14 dishes and genomic DNA from 2 dishes were isolated and sequenced. Of the input genomic DNA, 99.71% was recovered in the isolated DNA, and 99.89% of the corresponding RNA was detected above a threshold of 10 tags. The mean number of tags per bin (10 bp) was 145.5 in DNA sequencing and 140.3 in RNA sequencing, with SDs of 48.9 and 125.1, respectively (Figure 2A and 2B).

The log2 fold change (L2FC) of RNA reads over DNA reads was calculated for reads with >20 tags in the RNA sequencing data. Peak calling was performed with a threshold of 0.8 L2FC (corresponding to a 1.74-fold change in expression) with a 50-bp bin and a 20-tag cutoff, combining adjoining peaks within this 50-bp bin. In total, we obtained 197 regions encompassing 60 600 bp of candidate REs with transcription-enhancing capacity (Tables II and III in the Data Supplement). On average, each BAC possessed 13 RE regions with an average total peak length of 4097 bp. Each of the loci spanning *KCNN3*, *SCN10A*, and *WNT8A* possessed only 2 regions displaying more than a 1.74-fold change in expression. With 37 regions showing a fold change >1.74, the *GJA1* locus had the highest number of hits. As the promoter in the human pSTARR-seq used to identify REs has been reported to display relatively high basal activity,[24] we hypothesized that this property could be used to detect REs with repressing activity. We analyzed regions displaying a higher number of reads in the DNA than in the RNA, finding 248 regions with an L2FC <—0.8 with a 50-bp bin, combining adjoining peaks within this 50-bp bin (Tables II and III in the Data Supplement). Together, these regions span 46 150 bp of candidate REs with repressing capacity, in line with observations of others.[25]

As a proof of concept for our STARR-seq screen, we confirmed the activity for previously reported cardiac enhancers intronic of *SCN5A*[26] and *SCN10A*[27] (Figure 2C). Interestingly, around promoters, we observed a negative L2FC, indicating that a promoter sequence, when inserted downstream of the SCP1 promoter of the STARR-seq plasmid, interferes with transcription and

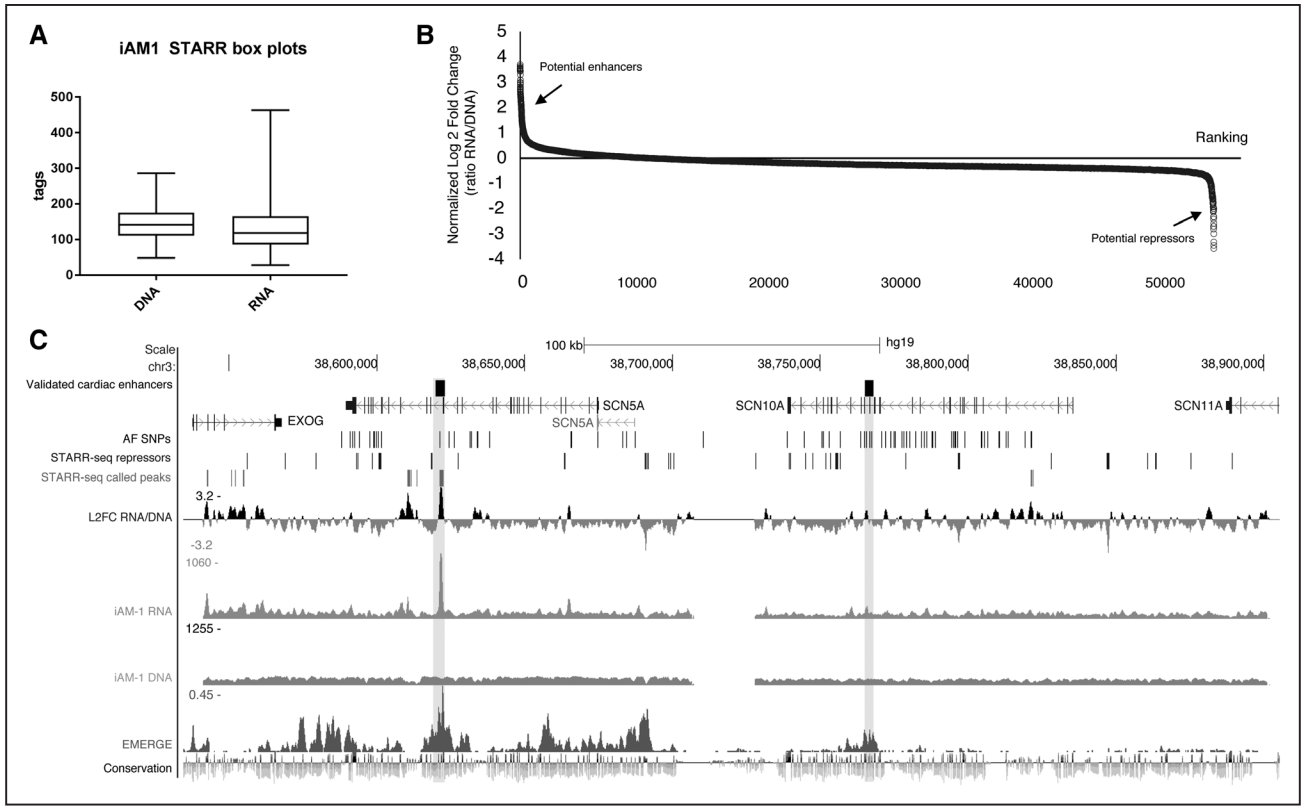

Figure 2. Self-transcribing active regulatory region sequencing (STARR-seq) output identifies regulatory elements.
A, Tag distribution of DNA-seq and RNA-seq in inducible atrial cardiomyocyte (iAM)-1 STARR-seq. **B**, Log2 fold change (L2FC) of RNA over DNA reads showing that a fraction of regions is overrepresented in the RNA (L2FC>0.8, potential enhancers) and a fraction is enriched in the DNA (L2FC<−0.8, potential repressors). **C**, *SCN5A/10A* locus showing atrial fibrillation (AF)-associated variants, called STARR-seq peaks (L2FC>0.8), L2FC of RNA over DNA reads, iAM-1 RNA-seq, iAM-1 DNA-seq, EMERGE output, and conservation track. The 2 areas highlighted in gray correspond to validated cardiac enhancers, both of which show STARR-seq signals. Promoter regions are shown as zoom-in underneath the genome browser of the University of California, Santa Cruz (UCSC) track. EXOG indicates Exo/Endonuclease G; and SNP, single-nucleotide polymorphism.

does not have strong enhancer activity (Figure 2C).[24] Examples are the promoter of an isoform of *SCN5A* and the region upstream of *SCN10A* (Figure 2C).

To identify putative cardiac TF-binding sites in the identified enhancer elements based on their signature consensus binding sequences, a TF-binding motif analysis was performed on the STARR-seq–called peaks with L2FC >1.5 corresponding to >2.8-fold increase in RNA reads over DNA reads using HOMER (Hypergeometric Optimization of Motif Enrichment).[28] Enriched motifs included recognition sites for MEF2 (myocyte enhancer factor 2) family members, NKX2-5 (NK2 homeobox 5), ESRRB (estrogen-related receptor-beta), COUP-TFII (COUP transcription factor 2), KLF5 (Krüppel-like factor 5), and MEIS2 (Meis homeobox 2; Figure 3A and 3C) with established roles in cardiac (atrial) development and transcription.[29–36] The enrichment of these recognition sites in the regions of enhanced transcription identified in this assay could reflect the enrichment of cardiac enhancers present in the library. Interestingly, recognition sites for IRFs (interferon regulatory factors) were highly represented in the top 10 enriched motifs (Figure 3A and 3B). Using RNA sequencing, we found expression of IRFs in human left atrial tissue (Figure IVC in the Data Supplement),[37] as well as in the iAM-1 cells in the absence of transfected DNA (Figure IVA and IVB in the Data Supplement). This may lead to the excess activation of fragments containing IRF motifs. However, the occurrence of IRF motifs could also be caused by an innate immune response due to plasmid transfection. As a negative control, we ran a similar analysis on randomly chosen STARR-seq regions of similar length with an L2FC between −1.5 and 1.5, revealing no enrichment of any relevant motifs (Figure 3F). When we analyzed the enrichment on the STARR-seq–called peaks of L2FC<−1.5 corresponding to a 2.8-fold decrease of RNA over DNA reads, we similarly found significant enrichment of MEF2 and NKX2 family members and other cardiac TFs, confirming the presence of cardiac-specific REs, representing potential repressors of expression (Figure 3D and 3E). For example, NKX2.5-binding motifs were found in 25% of sites, with multiple testing corrected Q value of 0.0001 and $P<10^{-5}$.

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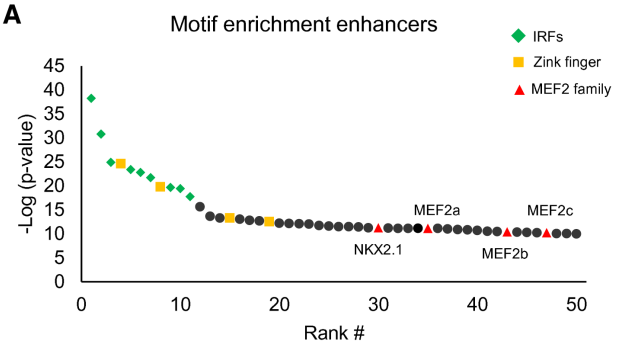

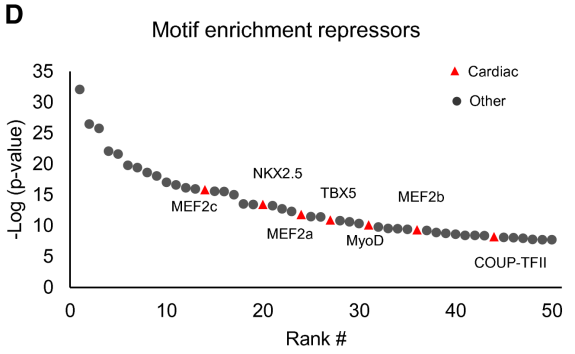

B

Top 10 motifs STARR-seq >1.5 L2FC

Motif rank	Motif Name	Consensus	P-value	% sites	q-value
1	IRF8		10^{-16}	16.75%	$<10^{-4}$
2	IRF1		10^{-13}	13.20%	$<10^{-4}$
3	IRF2		10^{-10}	11.17%	$<10^{-4}$
4	ZFX		10^{-10}	16.75%	$<10^{-4}$
5	IRF3		10^{-10}	16.24%	$<10^{-4}$
6	ISRE		10^{-09}	8.12%	$<10^{-4}$
7	IRF		10^{-09}	24.37%	$<10^{-4}$
8	ZNF711		10^{-08}	17.77%	$<10^{-4}$
9	IRF4		10^{-08}	15.23%	$<10^{-4}$
10	IRF8		10^{-08}	9.64%	$<10^{-4}$

E

Cardiac motifs enriched in STARR-seq <-1.5 L2FC

Motif rank	Motif Name	Consensus	P-value	% sites	q-value
15	Mef2c		10^{-06}	8.91%	$<10^{-4}$
18	Nkx2.2		10^{-06}	26.72%	$<10^{-4}$
21	Nkx2.5		10^{-05}	25.91%	$1x10^{-4}$
25	Mef2a		10^{-05}	8.10%	$3x10^{-4}$
26	Nkx2.1		10^{-04}	29.15%	$4x10^{-4}$
28	Tbx5		10^{-04}	30.77%	$6x10^{-4}$
32	MyoD		10^{-04}	6.07%	$1x10^{-3}$
37	Mef2b		10^{-04}	10.12%	$2x10^{-3}$
42	KLF5		10^{-03}	15.38%	$5x10^{-3}$
45	COUP-TFII		10^{-03}	17.00%	$6x10^{-3}$

C

Cardiac motifs enriched in STARR-seq >1.5 L2FC

Motif rank	Motif Name	Consensus	P-value	% sites	q-value
30	Nkx2.1		10^{-04}	29.95%	$4x10^{-4}$
35	Mef2a		10^{-04}	16.75%	$4x10^{-4}$
43	Mef2b		10^{-04}	24.87%	$7x10^{-4}$
47	Mef2c		10^{-04}	17.77%	$7x10^{-4}$
101	Nkx2.5		10^{-03}	24.37%	$5x10^{-3}$
112	Esrrb		10^{-03}	7.61%	$7x10^{-3}$
120	COUP-TFII		10^{-02}	12.18%	$8x10^{-3}$
153	KLF5		10^{-02}	5.08%	$1x10^{-2}$
158	Mef2d		10^{-02}	9.14%	$1x10^{-2}$
161	Meis1		10^{-02}	15.23%	$2x10^{-4}$

F

Top 10 STARR-seq motifs negative control

Motif rank	Motif Name	Consensus	P-value	% sites	q-value
1	Bapx1		10^{-02}	8.48%	0.98
2	Znf263		10^{-02}	4.02%	0.98
3	AP-2α		10^{-02}	1.79%	0.98
4	ARE		10^{-02}	1.79%	0.98
5	AP-2γ		10^{-02}	3.57%	0.98
6	MITF		10^{-02}	4.46%	0.98
7	WIP5		10^{-02}	3.57%	0.98
8	Slug		10^{-02}	1.79%	0.98
9	ANAC011		10^{-02}	1.79%	0.98
10	WRKY22		10^{-02}	3.12%	0.98

Figure 3. Transcription factor motif enrichment in self-transcribing active regulatory region sequencing (STARR-seq) identified elements.

A, Top 50 identified motifs in STARR-seq >1.5 log2 fold change (L2FC). **B**, Top 10 motifs in STARR-seq regions with >1.5 L2FC showing a strong representation of IRF (interferon regulatory factors)-binding sites. **C**, All cardiac-specific motifs enriched in the STARR-seq regions with L2FC >1.5. **D**, Top 50 TF (transcription factor) motifs identified by HOMER (Hypergeometric Optimization of Motif Enrichment) in STARR-seq <−1.5 L2FC and $P<0.05$. Motif rank is based on significance; %sites indicates percentage of STARR-seq <−1.5 L2FC containing this motif. **E**, All cardiac-specific motifs enriched in the STARR-seq regions with L2FC<−1.5. **F**, Top 10 motifs found in STARR-seq between −1.5 and 1.5 L2FC that we consider not to be potential regulatory elements. **A**–**F**, HOMER calculates *Q* values using the Benjamini-Hochberg false discovery rate procedure. *Q* values of <0.05 were considered significant. COUP-TFII indicates COUP transcription factor 2; MEF2, myocyte enhancer factor 2; MyoD, myoblast determination protein 1; NKX2-5, NK2 homeobox 5; and TBX5, t-box transcription factor 5.

Allele-Specific STARR-seq of AF-Associated Variants With Enhancer Potential

We next identified regulatory regions containing AF-associated SNPs. Such SNPs could allele specifically alter RE activity and could, therefore, add to the risk of AF in patients with this risk allele. To select identified atrial REs containing AF-associated variants, we intersected AF variants and the STARR-seq output. Because the STARR-seq tests transcriptional regulatory activity of episomal (ie, nonchromosomally integrated) DNA fragments outside their chromatin context, they may report the activity of DNA fragments that are not active in vivo.[24] To address this issue and to better discriminate between false and real positive signals, we cross-referenced our data using EMERGE.[38] This is a bioinformatics tool that integrates cardiac-specific ATAC (assay for transposase-accessible chromatin) sequencing data, histone marks, and various TF chromatin immunoprecipitation sequencing data for tissue-specific enhancer prediction (Figure 4A). We selected 43 regions with overlapping STARR-seq signal, EMERGE signal, and AF-associated variants, containing a total of 78 variants with a threshold of $P<10^{-4}$ (Table I in the Data Supplement). We chose

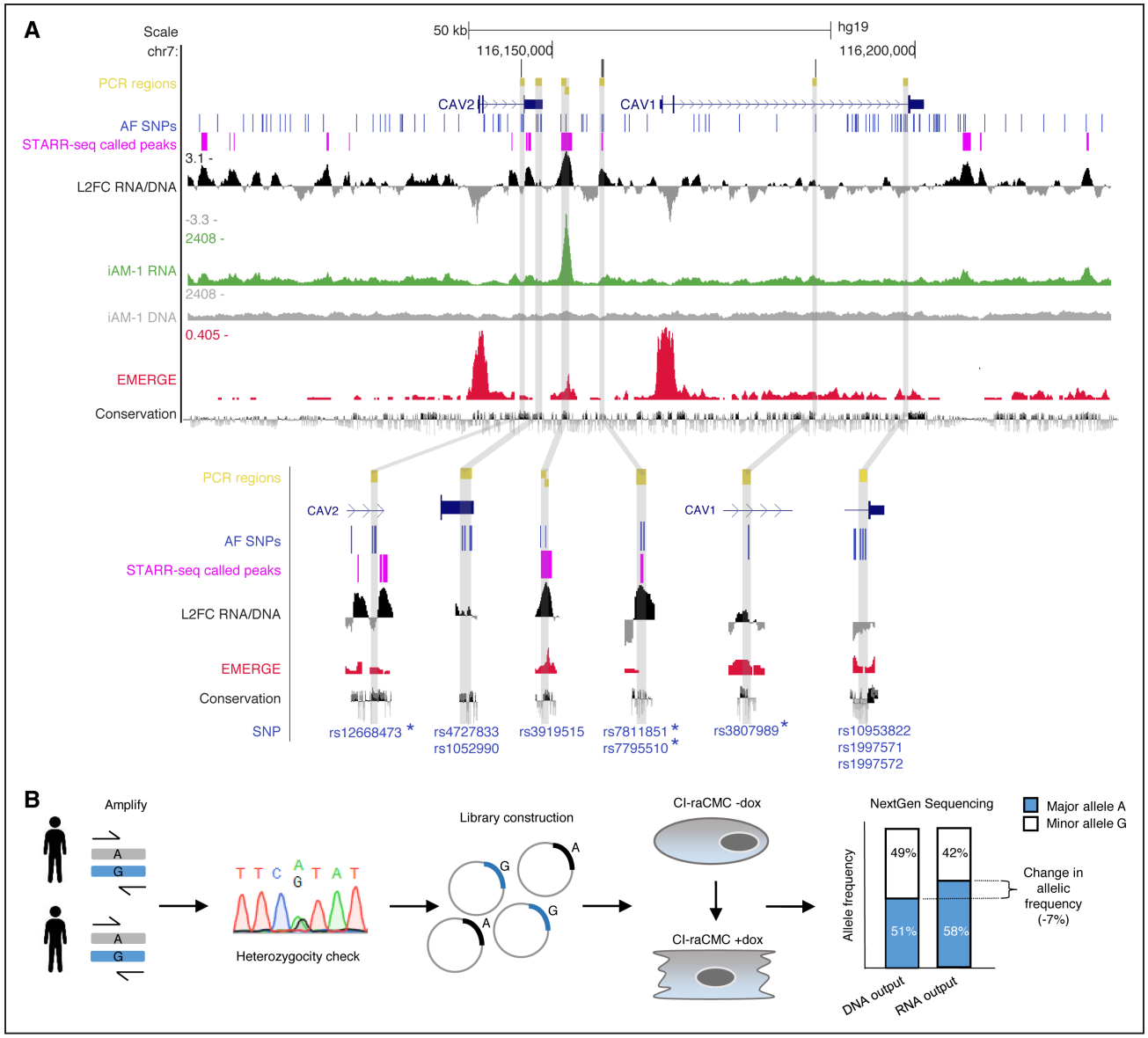

Figure 4. Variant self-transcribing active regulatory region sequencing (STARR-seq) shows significant allele-specific regulatory potential.

A, *CAV1/CAV2* locus showing atrial fibrillation (AF) variants, STARR-seq called peaks, log2 fold change (L2FC) of RNA over DNA reads, inducible atrial cardiomyocyte (iAM)-1 RNA sequencing (seq), iAM-1 DNA-seq, EMERGE output, and conservation track. Highlighted areas are regions included in the variant STARR-seq. Zoom-in of the regions included in the variant STARR-seq, with variant name. Star indicates significant difference in STARR-seq reads between minor and major alleles. **B**, Method of the variant STARR-seq. Regions of 400 to 900 bp including the selected variants were amplified from several patients. Regions were checked for heterozygosity by Sanger sequencing. Next, a library was constructed and transfected into iAM-1 cells. After cardiomyogenic differentiation of these cells, DNA and RNA were extracted followed by DNA-seq and RNA-seq. Allele frequencies in DNA were compared with RNA. CI-raCMC indicates conditionally immortalized rat atrial myocytes; PCR, polymerase chain reaction; and SNP, single-nucleotide polymorphism.

this threshold based on observations that subthreshold variants (association $P<10^{-4}$) can affect RE activity and may represent true risk variants.[39]

Primers were designed for the selected regions, typically amplifying 450- to 900-bp fragments containing all assigned variants. To amplify the selected variant enhancers, a database containing whole genome sequencing data of AF patients was searched for samples heterozygous for the respective variants, and selected variants were amplified using this input DNA library (Figure 4B). The presence of both alleles of the selected variants was verified using Sanger sequencing, and the fragments were combined in equimolar amounts. Subsequently, these fragments were inserted into pSTARR-seq_human, and the resulting reporter plasmid library was transfected into iAM-1 cells. After 9 days of differentiation, DNA and RNA were extracted from the cells and sequenced. Of the 78 selected variants, 72 were detected after DNA

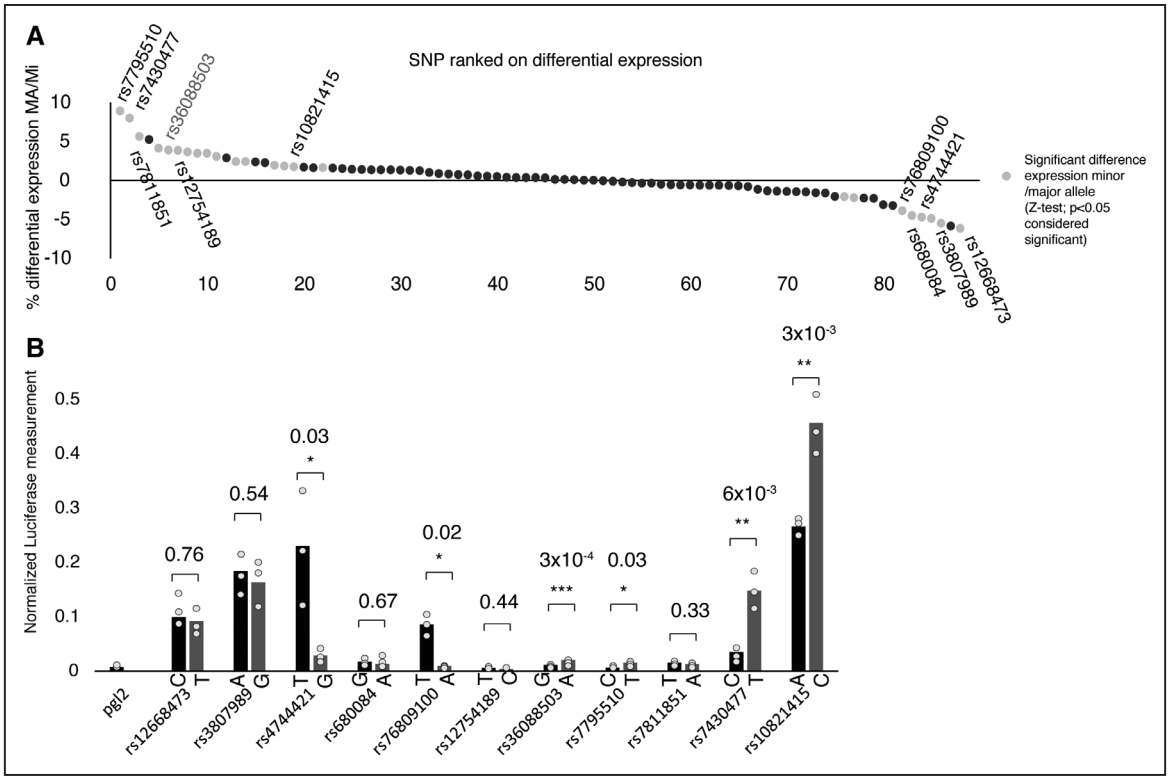

Figure 5. Variant self-transcribing active regulatory region sequencing (STARR-seq) and luciferase assay identify variants causing regulatory element differential activity.

A, Overview of all 88 variants showing differential expression (expressed in percentage) of the major allele (MA) over the minor allele (Mi) in the variant STARR-seq assay. The 24 significantly different variants are indicated by green dots. Significance was assessed by *Z* test; $P<0.05$ was considered significant. **B**, Luciferase assay showing normalized measurements of the transcriptional activity conferred by the MA and Mi of atrial fibrillation (AF) variants. SNP indicates single-nucleotide polymorphism. *$P<0.05$ was considered significant (as assessed by 2-tailed Student *t* test), **$P<0.01$, ***$P<0.001$.

sequencing (92.3%; Table IV in the Data Supplement). We quantified the number of reads of both alleles of the variants in DNA and RNA sequencing using DESeq2 and a *Z* test to compare the proportion of each allele in the transfected (DNA input library) relative to its counterpart in the transcribed material (RNA output library). This analysis showed that 24 variants had significantly different allelic ratios between the input and output library, indicative of altered regulatory activity (33.3%; Table V in the Data Supplement; Figure 5A and 5B). The significant differential activities ranged from 1.6% to 8.8% (Table V in the Data Supplement).

To further validate the allele-specific enhancer activity, we tested a number of these variant REs by luciferase assay in the iAM-1 cell line. Comparison of normalized major and minor allele luciferase values revealed that 5 of 10 variants showed a significant difference in activity (Figure 5B). One of these variants, rs7430477 T/C lies within a previously identified enhancer intronic of *SCN10A*.[27] The direction of the change is consistent with the allele-specific STARR-seq results for 3 of 5 of the significant variants in the luciferase assay: rs36088503, rs7430477, and rs10821415. The other variants (rs7795510 and rs76809100) show a higher activity for the allele that showed a lower activity in the STARR-seq.

To look into the potential mechanism by which the variants affect DNA transcription, find individual motif occurrence was performed on a 20-bp sequence window around the 24 variants with allele-specific function.[40] This analysis showed 6 SNPs to alter potential TF-binding sites (Table VI in the Data Supplement). One of these TF-binding site–altering variants is rs6495063. The minor allele of this variant (T; allele frequency, 22%) changes the DNA sequence to a greatly improved predicted recognition site for the TF ESRRB, potentially leading to stronger binding of the DNA.

We next asked which genes are most likely regulated by these REs. To this end, we searched a promoter capture Hi-C data set performed in iPSC-derived cardiomyocytes.[21] Promoter capture Hi-C,[41,42] which is an adaptation of chromosome conformation capture technique Hi-C,[43] highly enriches for promoter sequence interactions. We interrogated our 24 functional AF variants for promoter interactions in this data set. A number of the variant-containing regions showed interaction with the promoter of one or several genes, giving an indication of the potential functional targets of the identified enhancers modified by these AF-associated

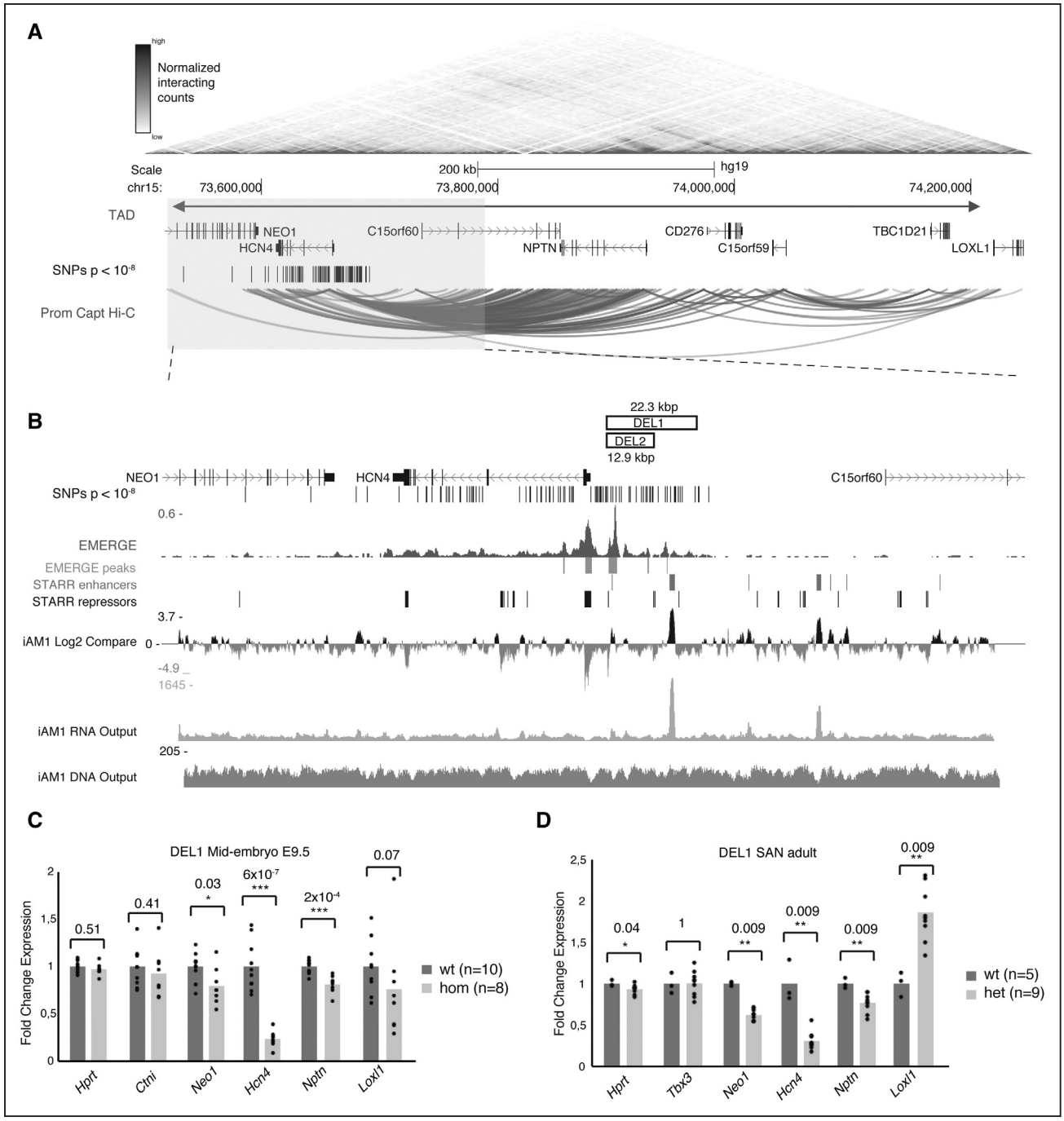

Figure 6. Identification of enhancer region in the Hcn4 locus in mice.

A, *HCN4* locus (GRCh37/hg19 assembly) showing the genes in the *HCN4* topologically associating domain (TAD; shown by Hi-C data in the top track, Rao et al[43a]), atrial fibrillation (AF)–associated variants, and promoter capture Hi-C (PCHi-C) data (bottom track, Montefiori et al[21]). **B**, Zoom-in of the region surrounding *HCN4* indicating the 22.4-kbp deletion of both the proximal and distal regulatory element (RE; DEL [deletion]-1) and the 12.9-kbp deletion of the proximal RE only (DEL2). Shown are the EMERGE output (RE prediction), called EMERGE peaks, self-transcribing active regulatory region (STARR) enhancers and repressors, STARR log2 fold change (L2FC) of RNA over DNA reads, STARR DNA sequencing (seq), and RNA-seq. **C**, RT-qPCR analysis of the effects of the AF-associated variants identified in the *HCN4* (potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4) locus on the transcription of potential target genes within the TAD of mouse *Hcn4* (significance assessed by Mann-Whitney *U* test). **D**, RT-qPCR demonstrating that deletion of DEL1$^{+/-}$ alters the expression of all genes tested in the TAD of *Hcn4* in sinoatrial node (SAN) tissue of adult mice (significance assessed by Mann-Whitney *U* test). NEO1 indicates Neogenin 1; and SNP, single-nucleotide polymorphism.

variants (Table VII in the Data Supplement). For example, in the *HCN4* locus, 2 variants (rs6495063 and rs6495062) showed allele-specific RE activity in the STARR-seq. Moreover, they are in contact with the promoter of *NPTN*, as determined by promoter capture Hi-C (Figure 6A), indicating this is a possible target gene of these REs.

In Vivo Analysis of STARR-seq Identified RE

For in vivo analysis, we selected a variant region with a strong STARR-seq signal overlapping an AF-associated variant for which no expression quantitative trait locus data are available. Using STARR-seq, we identified 2 potential REs on chr15q24.1 with lead SNP rs7172038, upstream of *HCN4* that contain AF-associated variants ($P<10^{-8}$). To assess the function of these REs and their target genes in vivo, we made 2 deletions in the mouse genome using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9) technology (Figure 6B). We deleted both the proximal and distal RE together (DEL [deletion]-1; mm9 chr9:58,644,674-58,667,035) and the proximal RE alone (DEL2; mm9 chr9:58,654,166-58,667,035). Both deletions encompass the lead SNP rs7172038 and the proximal RE, while only the large deletion contains the distal RE identified with STARR-seq. Both the single and double RE deletion mutants were homozygous lethal around embryonic day 11.5. Functional RE-target promoter interactions most frequently occur within the same topologically associating domain.[44–46] Therefore, to determine which genes could be affected by the deletions, we screened expression of all genes within the topologically associating domain. The future thoracic region of the embryo containing the heart including inflow tract, which selectively expresses *Hcn4* at this stage,[47] was tested at embryonic day 9.5 for expression of *Neo1*, *Hcn4*, *Nptn*, and *Loxl1* in DEL1$^{-/-}$ animals. We did not test *C15orf60* or *C15orf59* because these genes are not expressed in heart tissue (human or mouse).[37] Of these genes, only *Hcn4* was significantly downregulated (73%) in homozygotes (n=10) compared with wild-type littermates (n=8) as shown by Mann-Whitney *U* test (Figure 6C). As a control, the expression of the same genes was tested in the remaining tissue of the embryo, showing no significant differences as tested by Mann-Whitney *U* test ($P>0.001$) in expression between DEL1$^{-/-}$ and wild-type embryos (Figure VA in the Data Supplement).

The expression of the same genes was tested in microdissected sinoatrial node regions comparing adult DEL1$^{+/-}$ (n=9) with wild-type (n=3) animals. In the DEL1$^{+/-}$ sinoatrial node region, Hcn4 was downregulated as shown by Mann-Whitney *U* test (Figure 6D). Furthermore, in DEL1$^{+/-}$, expression of *Neo1* was decreased and *Loxl1* increased (Figure 6D). In contrast, in DEL2$^{+/-}$, expression of *Neo1* was slightly increased and of *Loxl1* slightly decreased (Figure VB in the Data Supplement). Collectively, these data indicate that within the topologically associating domain, *Hcn4* is the major target gene regulated by the proximal REs and that the region uniquely deleted in DEL1 additionally influences the regulation of *Neo1* and *Loxl1* (Figure 6).

Mice heterozygous for DEL1 presented with frequent sinus pauses (Figure 7A and 7B) similar to previous reports of conditional *Hcn4* knockouts,[48–50] with increased heart rate variability (Figure 7C), as well as fragmented QRS (Figure 7D and 7E; Figure VIA and VIB), and rare ectopic atrial activations. Male (n=7) and female (n=6) mice were used, both showing normal data distribution and no significant differences between the sexes as determined by 2-tailed *t* test. Additionally, we observed a modest increase in PR interval duration, whereas other ECG parameters remained unchanged (Figure 7F). To assay sinoatrial and atrioventricular node function, we performed transesophageal burst pacing and found that heterozygous mice showed a significantly longer cSNRT (corrected sinus node recovery time) at 100 and 120 ms pacing frequencies (Figure 7G), whereas atrioventricular node function remained unaffected (Figure 7H). Moreover, 2 of 14 heterozygous mice presented with over 1 second cumulative AF upon rapid atrial pacing stimuli (Figure 7I; Figure VIC and VID). These data reveal the critical role of a STARR-seq–identified RE for mouse development, *Hcn4* expression, and electrophysiology.

DISCUSSION

GWAS have identified a large number of variants (SNPs) associated with AF in over 100 loci.[11] Identification of the functional variants among these AF-associated SNPs has lagged, because the identification of biologically relevant REs and the impact of a variant on their function and on target gene regulation has remained technically challenging. Here, using STARR-seq and a recently generated line of differentiation-competent myocytes, we identified >400 potential REs in 12 AF-associated loci. Subsequently, we identified 24 variant REs with allele-specific regulatory activity using an adapted STARR-seq approach.

High-throughput reporter assays have associated candidate REs with unique barcodes, such as in massively parallel reporter assays,[51–53] or with the candidate REs themselves, such as in STARR-seq assays.[54] STARR-seq enables the use of discrete regions of larger stretches of genomic material in contrast to massively parallel reporter assays in which oligodeoxyribonucleotides are synthesized, restricting the length of the fragments that can be analyzed.[55] A drawback of both techniques is the use of episomal plasmid DNA elements to investigate the functionality of REs. Sequences with putative elements may be activated when tested episomally, but this does not represent the normal in vivo situation where the REs are subjected to chromatin packaging.[56] It is likely that the occurrence of the observed *Irf* recognition sites in the enriched motifs of the STARR-seq is caused by the transfection of this episomal plasmid DNA, which causes the induction of the innate immune response in the iAM-1 cells, also

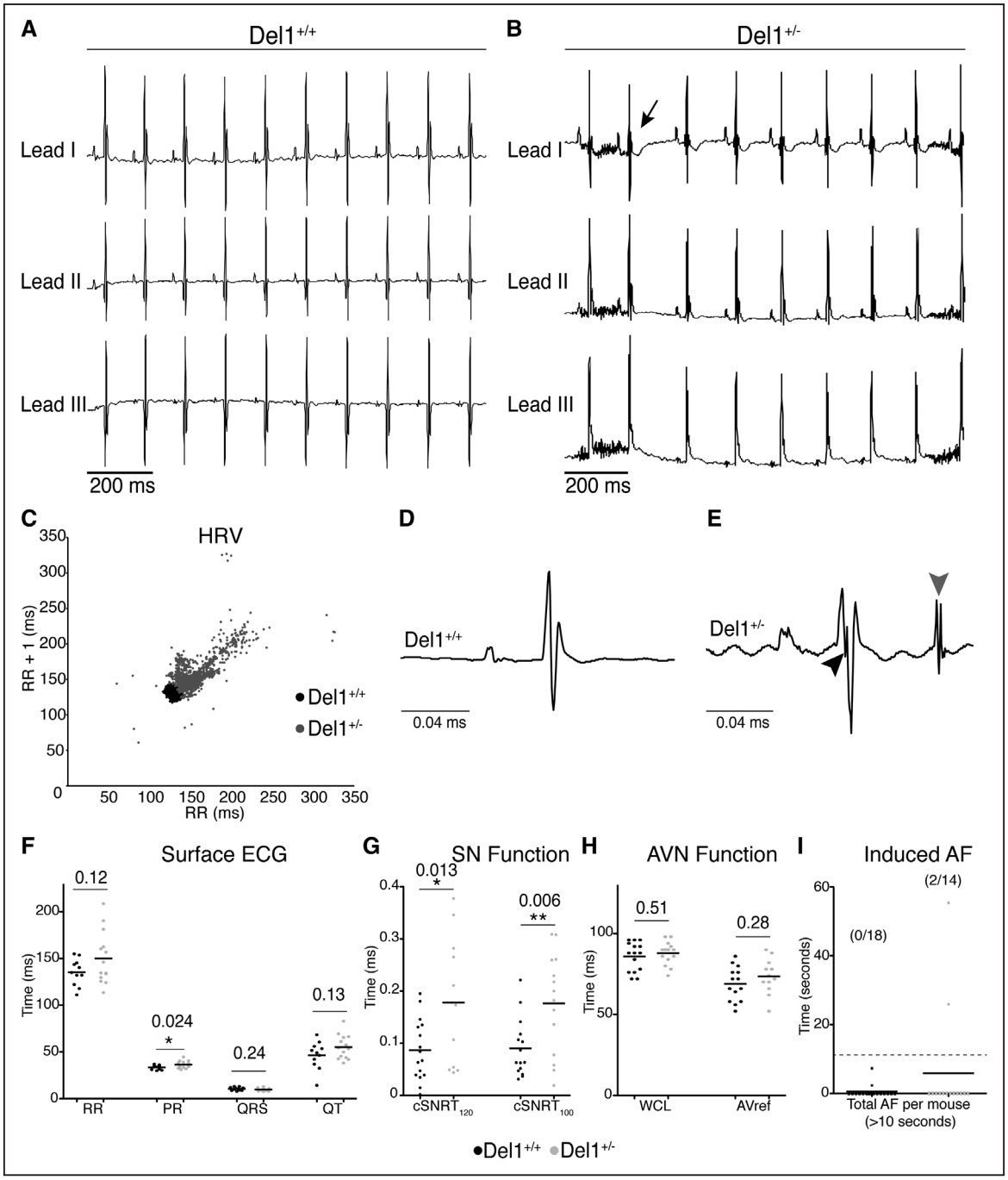

Figure 7. *Hcn4* regulatory element (RE) DEL (deletion)-1$^{+/-}$ mice present with heart rhythm abnormalities.
A and **B**, Representative 3-lead surface ECG traces of (**A**) WT and (**B**) DEL1$^{+/-}$ mice showing sinus pauzes after respiration (arrow). **C**, Representative Pointcaré plot of WT (DEL$^{+/+}$) and DEL1$^{+/-}$ individuals showing increased heart rate variability (HRV). **D**, Sample single ECG trace of a DEL1$^{+/+}$ (**left**) and (**E**) DEL1$^{+/-}$ (**right**) depicting a fragmented QRS (black arrowhead) and ectopic atrial activation (red arrowhead). **F**, Summary of surface ECG parameters. **G**, Plots of corrected sinus node recovery time (cSNRT) at 120 and 100 ms pacing as an indication of sinus node (SN) function. **H**, Plots of Wenckebach cycle length (WCL) and atrial-ventricular (AV) refractory times as an indication of atrioventricular node (AVN) function. **I**, Plot depicting cumulative time spent in atrial fibrillation (AF) after burst pacing per mouse. **F**–**H**, Exact *P* values are reported and calculated by 2-tailed Student *t* test. *$P<0.05$ was considered significant, **$P<0.01$.

observed by others.[57,58] To discriminate between bona fide functional REs and those that display activity only in reporter plasmids, we used EMERGE.[38] In this recently developed software package, existing (epi)genomic data sets are integrated and weighed, allowing us to evaluate candidate REs in the proper tissue-specific epigenomic context. Using available chromatin conformation data of differentiated cardiomyocytes,[21] the possible target genes of these REs were mapped. To identify its target genes and their physiological function,

we deleted the orthologue of an RE close to *HCN4* from the mouse genome, which led to selective loss of *Hcn4* expression in heterozygous mice, embryonic lethality of homozygous mutants, and arrhythmia in heterozygous mutant mice.

In our experience, the use of an appropriate cell line is crucial to find biologically meaningful results using STARR-seq. Others have used S2, ovarian somatic cells, and HeLa cells for STARR-seq assays,[19,59] but we used iAM-1 cells for identifying AF-associated REs as these conditionally immortalized atrial myocytes closely resemble primary atrial myocytes in both structure and function following their cardiomyogenic differentiation. This makes them highly suitable for our purpose, that is, the identification of atrial cardiomyocyte-specific variant enhancers.[20] However, the use of this specialized cell line also limits the analysis to cardiomyocytes, excluding any potentially relevant variant RE activity found in other cell types in the heart such as fibroblasts and endothelial cells. Another consideration is the relative (im)maturity of the currently available in vitro atrial cardiomyocyte models.[60,61] Neither pluripotent stem cell–derived atrial cardiomyocytes nor permanently immortalized atrial cardiomyocytes possess functional and structural characteristics similar to those of the primary cells from which they were derived. Extensive transcriptional, immunocytological, and electrophysiological analyses showed that the iAM-1 cell line used in this study outperforms all other cardiomyocyte lines including the often used HL-1 cell line in terms of cardiomyogenic differentiation ability.[20] Moreover, optical voltage mapping experiments of confluent iAM-1 monolayers revealed that the cells reach their maximum level of cardiomyogenic differentiation after 9 days (of culture in the absence of doxycycline). RT-qPCR analysis of the samples collected at differentiation day 0, 9, and 12 showed that all tested cardiomyocyte maturation markers increased significantly from day 0 to 9 of iAM-1 differentiation, but no significant additional increase was observed between days 9 and 12 of cardiomyogenic differentiation (Figure I in the Data Supplement).

Comparing RNA with DNA reads, we not only identified enhancers in the iAM-1 cell line using STARR-seq but also observed regions with transcription-lowering activity, potentially reflecting repressors. It is known that the STARR-seq vector as it was originally published[19] contains an SCP1 promoter that has a high basal activity and an origin of replication that could act as a conflicting core promoter, and that could potentially lead to elevated background activity in STARR-seq assays.[24,62,63] In this case, the basal activity combined with the tissue specificity appears to enable the identification of regions with decreased (repressive) transcriptional activity. The observed enrichment of cardiac TF-binding sites in these repressing regions strongly suggests that they represent bona fide cardiomyocyte-relevant REs. Thus, STARR-seq may be used for repressor identification, in line with observations of others.[25]

The observed difference in activity between STARR-seq–identified variant AF-associated SNPs was significant but modest, which may, in part, be due to the strong core promoter in the pSTARR vector and the position of the RE, downstream of the promoter, which blunt measured differences in enhancer activity[24] (our observations). Nevertheless, the observed small difference in activity of such variant REs is in line with the estimated small effect size of risk variants on the expression of nearby genes.[64] How this relatively mild change in enhancer activity due to single-nucleotide changes leads to AF is not well established. There are an estimated 10× more REs compared with genes, implying that a gene could be regulated by multiple REs.[65] Indeed, it has been shown that multiple enhancers are needed for the correct dynamic expression of certain genes throughout development.[66–68] This use of multiple RE or combinatorial RE interactions involving both repressors and enhancers could be mechanisms at play in the loci in this study, and it is possible that the activity of individual REs is influenced by such interactions in vivo, resulting in an overestimation of RE activity. However, the tools to investigate such interactions are currently lacking. On the other hand, some RE activity could also be missed in our assay, as multiple affected REs may synergistically lead to small but significant changes in expression of their target gene(s). Such a mechanism was proven to increase the risk of the complex disease Hirschsprung[69] and is likely to play a role in AF risk as well. Furthermore, the small effects seen in transfection-based assays may underestimate the effect size of the variant on RE function in vivo. REs usually function in the context of local genome structure, chromosome conformation, and epigenetic state. A change in TF binding (altered affinity, different TF) may strongly impact on endogenous transcriptional regulatory complexes while the consequences for artificial, episome-driven transcription as with pSTARR-seq human are small.

Variant RE-target gene identification requires functional assays in addition to contact maps. We identify 2 variants with allele-specific regulatory activity and here show an example of determining the functional targets. Deletion of the 2 REs identified by STARR-seq homologous to the human region upstream of *Hcn4* in the mouse in vivo led to homozygous lethality at embryonic day 11.5 and severely diminished *Hcn4* expression in heterozygous animals, showing that the deleted region(s) have indispensable developmental function. *Hcn4* expression is essential for proper generation of pacemaker potentials in the developing sinoatrial node, and global and cardiomyocyte-specific *Hcn4* depletion leads to embryonic lethality before embryonic day 11.5.[48–50,70] We see increased heart rate variability, sinus node dysfunction,

and AF inducibility in heterozygous mice, similar to observations in familial HCN4 mutations in human that cause both bradycardia and AF.[71,72] Moreover, studies suggest that bradycardia could be hidden by a reflex sinus tachycardia,[71,73] and that slow heart rate is a substrate for AF through the increased likelihood of atrial ectopy,[74,75] and thus causes increased risk of AF.[76]

Although we here identify gene targets of REs in vivo, we are currently still unable to address the function of SNPs since the effect sizes of disease-associated variants are expected to be small in most cases, which may not be detected in dedicated mouse models, rendering their generation and analysis laborious and risky. To evaluate such an effect, multiple—unknown—synergistic SNPs are likely needed in parallel. Moreover, the conservation of human REs in the mouse is only moderate,[77] rendering testing in human cells necessary. However, as of yet, it is technically not feasible to pick up small effects on gene expression in differentiated human stem cells, the only tool available to date, because of the variation in phenotype of differentiated cell types derived from different modified stem cell clones.

ARTICLE INFORMATION

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Disclosures

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Supplemental Materials

Expanded Materials and Methods
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REFERENCES

1. Staerk L, Sherer JA, Ko D, Benjamin EJ, Helm RH. Atrial fibrillation: epidemiology, pathophysiology, and clinical outcomes. *Circ Res*. 2017;120:1501–1517. doi: 10.1161/CIRCRESAHA.117.309732
2. Fox CS, Parise H, D'Agostino RB Sr, Lloyd-Jones DM, Vasan RS, Wang TJ, Levy D, Wolf PA, Benjamin EJ. Parental atrial fibrillation as a risk factor for atrial fibrillation in offspring. *JAMA*. 2004;291:2851–2855. doi: 10.1001/jama.291.23.2851
3. Lubitz SA, Sinner MF, Lunetta KL, Makino S, Pfeufer A, Rahman R, Veltman CE, Barnard J, Bis JC, Danik SP, et al. Independent susceptibility markers for atrial fibrillation on chromosome 4q25. *Circulation*. 2010;122:976–984. doi: 10.1161/CIRCULATIONAHA.109.886440
4. Nattel S, Dobrev D. Electrophysiological and molecular mechanisms of paroxysmal atrial fibrillation. *Nat Rev Cardiol*. 2016;13:575–590. doi: 10.1038/nrcardio.2016.118
5. Benjamin EJ, Rice KM, Arking DE, Pfeufer A, van Noord C, Smith AV, Schnabel RB, Bis JC, Boerwinkle E, Sinner MF, et al. Variants in ZFHX3 are associated with atrial fibrillation in individuals of European ancestry. *Nat Genet*. 2009;41:879–881. doi: 10.1038/ng.416
6. Ellinor PT, Lunetta KL, Glazer NL, Pfeufer A, Alonso A, Chung MK, Sinner MF, de Bakker PI, Mueller M, Lubitz SA, et al. Common variants in KCNN3 are associated with lone atrial fibrillation. *Nat Genet*. 2010;42:240–244. doi: 10.1038/ng.537
7. Ellinor PT, Lunetta KL, Albert CM, Glazer NL, Ritchie MD, Smith AV, Arking DE, Müller-Nurasyid M, Krijthe BP, Lubitz SA, et al. Meta-analysis identifies six new susceptibility loci for atrial fibrillation. *Nat Genet*. 2012;44:670–675. doi: 10.1038/ng.2261
8. Sinner MF, Tucker NR, Lunetta KL, Ozaki K, Smith JG, Trompet S, Bis JC, Lin H, Chung MK, Nielsen JB, et al. Integrating genetic, transcriptional, and functional analyses to identify five novel genes for atrial fibrillation. *Circulation*. 2014;130:1225–1235. doi: 10.1161/CIRCULATIONAHA.114.009892
9. Christophersen IE, Rienstra M, Roselli C, Yin X, Geelhoed B, Barnard J, Lin H, Arking DE, Smith AV, Albert CM, et al; METASTROKE Consortium of the ISGC; Neurology Working Group of the CHARGE Consortium; AFGen Consortium. Large-scale analyses of common and rare variants identify 12 new loci associated with atrial fibrillation. *Nat Genet*. 2017;49:946–952. doi: 10.1038/ng.3843
10. Low SK, Takahashi A, Ebana Y, Ozaki K, Christophersen IE, Ellinor PT, Ogishima S, Yamamoto M, Satoh M, Sasaki M, et al; AFGen Consortium. Identification of six new genetic loci associated with atrial fibrillation in the Japanese population. *Nat Genet*. 2017;49:953–958. doi: 10.1038/ng.3842
11. Roselli C, Chaffin MD, Weng LC, Aeschbacher S, Ahlberg G, Albert CM, Almgren P, Alonso A, Anderson CD, Aragam KG, et al. Multi-ethnic genome-wide association study for atrial fibrillation. *Nat Genet*. 2018;50:1225–1233. doi: 10.1038/s41588-018-0133-9
12. Nielsen JB, Thorolfsdottir RB, Fritsche LG, Zhou W, Skov MW, Graham SE, Herron TJ, McCarthy S, Schmidt EM, Sveinbjornsson G, et al. Biobank-driven genomic discovery yields new insight into atrial fibrillation biology. *Nat Genet*. 2018;50:1234–1239. doi: 10.1038/s41588-018-0171-3
13. Schaub MA, Boyle AP, Kundaje A, Batzoglou S, Snyder M. Linking disease associations with regulatory information in the human genome. *Genome Res*. 2012;22:1748–1759. doi: 10.1101/gr.136127.111
14. Kleinjan DJ, Coutinho P. Cis-ruption mechanisms: disruption of cis-regulatory control as a cause of human genetic disease. *Brief Funct Genomic Proteomic*. 2009;8:317–332. doi: 10.1093/bfgp/elp022
15. Maurano MT, Haugen E, Sandstrom R, Vierstra J, Shafer A, Kaul R, Stamatoyannopoulos JA. Large-scale identification of sequence variants influencing human transcription factor occupancy in vivo. *Nat Genet*. 2015;47:1393–1401. doi: 10.1038/ng.3432
16. Krijger PH, de Laat W. Regulation of disease-associated gene expression in the 3D genome. *Nat Rev Mol Cell Biol*. 2016;17:771–782. doi: 10.1038/nrm.2016.138
17. Tucker NR, Ellinor PT. Emerging directions in the genetics of atrial fibrillation. *Circ Res*. 2014;114:1469–1482. doi: 10.1161/CIRCRESAHA.114.302225
18. Roselli C, Chaffin MD, Weng LC, Aeschbacher S, Ahlberg G, Albert CM, Almgren P, Alonso A, Anderson CD, Aragam KG, et al. Multi-ethnic genome-wide association study for atrial fibrillation. *Nat Genet*. 2018;50:1225–1233. doi: 10.1038/s41588-018-0133-9
19. Arnold CD, Gerlach D, Stelzer C, Boryń ŁM, Rath M, Stark A. Genome-wide quantitative enhancer activity maps identified by STARR-seq. *Science*. 2013;339:1074–1077. doi: 10.1126/science.1232542
20. Liu J, Volkers L, Jangsangthong W, Bart CI, Engels MC, Zhou G, Schalij MJ, Ypey DL, Pijnappels DA, de Vries AAF. Generation and primary

characterization of iAM-1, a versatile new line of conditionally immortalized atrial myocytes with preserved cardiomyogenic differentiation capacity. *Cardiovasc Res*. 2018;114:1848–1859. doi: 10.1093/cvr/cvy134
21. Montefiori LE, Sobreira DR, Sakabe NJ, Aneas I, Joslin AC, Hansen GT, Bozek G, Moskowitz IP, McNally EM, Nobrega MA. A promoter interaction map for cardiovascular disease genetics. *eLife*. 2018;7:340869. doi: 10.7554/eLife.35788
22. Sander JD, Maeder ML, Reyon D, Voytas DF, Joung JK, Dobbs D. ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool. *Nucleic Acids Res*. 2010;38:W462–W468. doi: 10.1093/nar/gkq319
23. Muerdter F, Boryń ŁM, Arnold CD. STARR-seq - principles and applications. *Genomics*. 2015;106:145–150. doi: 10.1016/j.ygeno.2015.06.001
24. Muerdter F, Boryń ŁM, Woodfin AR, Neumayr C, Rath M, Zabidi MA, Pagani M, Haberle V, Kazmar T, Catarino RR, et al. Resolving systematic errors in widely used enhancer activity assays in human cells. *Nat Methods*. 2018;15:141–149. doi: 10.1038/nmeth.4534
25. Liu S, Liu Y, Zhang Q, Wu J, Liang J, Yu S, Wei GH, White KP, Wang X. Systematic identification of regulatory variants associated with cancer risk. *Genome Biol*. 2017;18:194. doi: 10.1186/s13059-017-1322-z
26. van der Harst P, van Setten J, Verweij N, Vogler G, Franke L, Maurano MT, Wang X, Mateo Leach I, Eijgelsheim M, Sotoodehnia N, et al. 52 genetic loci influencing myocardial mass. *J Am Coll Cardiol*. 2016;68:1435–1448. doi: 10.1016/j.jacc.2016.07.72
27. Van Den BM, Smemo S, Burnicka-Turek O, Arnolds DE, van de Werken HJ, Klous P, McKean D, Muehlschlegel JD, Moosmann J, Toka O, et al. A common genetic variant within *SCN10A* modulates cardiac *SCN5A* expression. *J Clin Invest*. 2014;124:1844–1852. doi: 10.1172/JCI73140
28. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh H, Glass CK. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell*. 2010;38:576–589. doi: 10.1016/j.molcel.2010.05.004
29. Desjardins C, Naya F. The function of the MEF2 family of transcription factors in cardiac development, cardiogenomics, and direct reprogramming. *J Cardiovasc Dev Dis*. 2016;3:26. doi: 10.3390/jcdd3030026
30. Harvey RP, Lai D, Elliott D, Biben C, Solloway M, Prall O, Stennard F, Schindeler A, Groves N, Lavulo L, et al. Homeodomain factor NKX2-5 in heart development and disease. *Cold Spring Harb Symp Quant Biol*. 2002;67:107–114. doi: 10.1101/sqb.2002.67.107
31. Shen T, Aneas I, Sakabe N, Dirschinger RJ, Wang G, Smemo S, Westlund JM, Cheng H, Dalton N, Gu Y, et al. Tbx20 regulates a genetic program essential to adult mouse cardiomyocyte function. *J Clin Invest*. 2011;121:4640–4654. doi: 10.1172/JCI59472
32. Rowe GC, Asimaki A, Graham EL, Martin KD, Margulies KB, Das S, Saffitz J, Arany Z. Development of dilated cardiomyopathy and impaired calcium homeostasis with cardiac-specific deletion of ESRRβ. *Am J Physiol Heart Circ Physiol*. 2017;312:H662–H671. doi: 10.1152/ajpheart.00446.2016
33. Wu SP, Kao CY, Wang L, Creighton CJ, Yang J, Donti TR, Harmancey R, Vasquez HG, Graham BH, Bellen HJ, et al. Increased COUP-TFII expression in adult hearts induces mitochondrial dysfunction resulting in heart failure. *Nat Commun*. 2015;6:8245. doi: 10.1038/ncomms9245
34. Drosatos K, Pollak NM, Pol CJ, Ntziachristos P, Willecke F, Valenti MC, Trent CM, Hu Y, Guo S, Aifantis I, et al. Cardiac myocyte KLF5 regulates ppara expression and cardiac function. *Circ Res*. 2016;118:241–253. doi: 10.1161/CIRCRESAHA.115.306383
35. Louw JJ, Corveleyn A, Jia Y, Hens G, Gewillig M, Devriendt K. MEIS2 involvement in cardiac development, cleft palate, and intellectual disability. *Am J Med Genet A*. 2015;167A:1142–1146. doi: 10.1002/ajmg.a.36989
36. He A, Kong SW, Ma Q, Pu WT. Co-occupancy by multiple cardiac transcription factors identifies transcriptional enhancers active in heart. *Proc Natl Acad Sci USA*. 2011;108:5632–5637. doi: 10.1073/pnas.1016959108
37. van Ouwerkerk AF, Bosada FM, van Duijvenboden K, Hill MC, Montefiori LE, Scholman KT, Liu J, de Vries AAF, Boukens BJ, Ellinor PT, et al. Identification of atrial fibrillation associated genes and functional non-coding variants. *Nat Commun*. 2019;10:4755. doi: 10.1038/s41467-019-12721-5
38. van Duijvenboden K, de Boer BA, Capon N, Ruijter JM, Christoffels VM. EMERGE: a flexible modelling framework to predict genomic regulatory elements from genomic signatures. *Nucleic Acids Res*. 2016;44:e42. doi: 10.1093/nar/gkv1144
39. Wang X, Tucker NR, Rizki G, Mills R, Krijger PHL, De Wit E, Subramanian V, Bartell E, Nguyen XX, Ye J, et al. Discovery and validation of sub-threshold genome-wide association study loci using epigenomic signatures. *Elife*. 2016;5:1–24. doi: 10.7554/eLife.10557
40. Grant CE, Bailey TL, Noble WS. FIMO: scanning for occurrences of a given motif. *Bioinformatics*. 2011;27:1017–1018. doi: 10.1093/bioinformatics/btr064
41. Schoenfelder S, Sugar R, Dimond A, Javierre BM, Armstrong H, Mifsud B, Dimitrova E, Matheson L, Tavares-Cadete F, Furlan-Magaril M, et al. Polycomb repressive complex PRC1 spatially constrains the mouse embryonic stem cell genome. *Nat Genet*. 2015;47:1179–1186. doi: 10.1038/ng.3393
42. Mifsud B, Tavares-Cadete F, Young AN, Sugar R, Schoenfelder S, Ferreira L, Wingett SW, Andrews S, Grey W, Ewels PA, et al. Mapping long-range promoter contacts in human cells with high-resolution capture Hi-C. *Nat Genet*. 2015;47:598–606. doi: 10.1038/ng.3286
43. Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO, et al. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science*. 2009;326:289–293. doi: 10.1126/science.1181369
43a. Rao SSP, Huntley MH, Durand NC, Stamenova EK. A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. *Cell*. 2014;159:1665–1680. doi 10.1016/j.cell.2014.11.021
44. Dixon JR, Selvaraj S, Yue F, Kim A, Li Y, Shen Y, Hu M, Liu JS, Ren B. Topological domains in mammalian genomes identified by analysis of chromatin interactions. *Nature*. 2012;485:376–380. doi: 10.1038/nature11082
45. Sexton T, Yaffe E, Kenigsberg E, Bantignies F, Leblanc B, Hoichman M, Parrinello H, Tanay A, Cavalli G. Three-dimensional folding and functional organization principles of the Drosophila genome. *Cell*. 2012;148:458–472. doi: 10.1016/j.cell.2012.01.010
46. Nora EP, Lajoie BR, Schulz EG, Giorgetti L, Okamoto I, Servant N, Piolot T, van Berkum NL, Meisig J, Sedat J, et al. Spatial partitioning of the regulatory landscape of the X-inactivation centre. *Nature*. 2012;485:381–385. doi: 10.1038/nature11049
47. Garcia-Frigola C, Shi Y, Evans SM. Expression of the hyperpolarization-activated cyclic nucleotide-gated cation channel HCN4 during mouse heart development. *Gene Expr Patterns*. 2003;3:777–783. doi: 10.1016/s1567-133x(03)00125-x
48. Herrmann S, Stieber J, Ludwig A. Pathophysiology of HCN channels. *Pflugers Arch*. 2007;454:517–522. doi: 10.1007/s00424-007-0224-4
49. Harzheim D, Pfeiffer KH, Fabritz L, Kremmer E, Buch T, Waisman A, Kirchhof P, Kaupp UB, Seifert R. Cardiac pacemaker function of HCN4 channels in mice is confined to embryonic development and requires cyclic AMP. *EMBO J*. 2008;27:692–703. doi: 10.1038/emboj.2008.3
50. Hoesl E, Stieber J, Herrmann S, Feil S, Tybl E, Hofmann F, Feil R, Ludwig A. Tamoxifen-inducible gene deletion in the cardiac conduction system. *J Mol Cell Cardiol*. 2008;45:62–69. doi: 10.1016/j.yjmcc.2008.04.008
51. Tewhey R, Kotliar D, Park DS, Liu B, Winnicki S, Reilly SK, Andersen KG, Mikkelsen TS, Lander ES, Schaffner SF, et al. Direct identification of hundreds of expression-modulating variants using a multiplexed reporter assay. *Cell*. 2016;165:1519–1529. doi: 10.1016/j.cell.2016.04.027
52. Ulirsch JC, Nandakumar SK, Wang L, Giani FC, Zhang X, Rogov P, Melnikov A, McDonel P, Do R, Mikkelsen TS, et al. Systematic functional dissection of common genetic variation affecting red blood cell traits. *Cell*. 2016;165:1530–1545. doi: 10.1016/j.cell.2016.04.048
53. Zhang P, Xia JH, Zhu J, Gao P, Tian YJ, Du M, Guo YC, Suleman S, Zhang Q, Kohli M, et al. High-throughput screening of prostate cancer risk loci by single nucleotide polymorphisms sequencing. *Nat Commun*. 2018;9:2022. doi: 10.1038/s41467-018-04451-x
54. Catarino RR, Stark A. Assessing sufficiency and necessity of enhancer activities for gene expression and the mechanisms of transcription activation. *Genes Dev*. 2018;32:202–223. doi: 10.1101/gad.310367.117
55. Spicuglia S, Santiago-Algarra D, Dao LTM, Pradel L, España A. Recent advances in high-throughput approaches to dissect enhancer function. *F1000Research*. 2017;6:939. doi: 10.12688/f1000research.11581.1
56. van Arensbergen J, Pagie L, FitzPatrick VD, de Haas M, Baltissen MP, Comoglio F, van der Weide RH, Teunissen H, Võsa U, Franke L, et al. High-throughput identification of human SNPs affecting regulatory element activity. *Nat Genet*. 2019;51:1160–1169. doi: 10.1038/s41588-019-0455-2
57. Dao LTM, Galindo-Albarrán AO, Castro-Mondragon JA, Andrieu-Soler C, Medina-Rivera A, Souaid C, Charbonnier G, Griffon A, Vanhille L, Stephen T, et al. Genome-wide characterization of mammalian promoters with distal enhancer functions. *Nat Genet*. 2017;49:1073–1081. doi: 10.1038/ng.3884
58. Wang X, He L, Goggin SM, Saadat A, Wang L, Sinnott-Armstrong N, Claussnitzer M, Kellis M. High-resolution genome-wide functional dissection of transcriptional regulatory regions and nucleotides in human. *Nat Commun*. 2018;9:5380. doi: 10.1038/s41467-018-07746-1

59. Yáñez-Cuna JO, Arnold CD, Stampfel G, Boryń LM, Gerlach D, Rath M, Stark A. Dissection of thousands of cell type-specific enhancers identifies dinucleotide repeat motifs as general enhancer features. *Genome Res*. 2014;24:1147–1156. doi: 10.1101/gr.169243.113
60. Karakikes I, Ameen M, Termglinchan V, Wu JC. Human induced pluripotent stem cell-derived cardiomyocytes: insights into molecular, cellular, and functional phenotypes. *Circ Res*. 2015;117:80–88. doi: 10.1161/CIRCRESAHA.117.305365
61. Bedada FB, Wheelwright M, Metzger JM. Maturation status of sarcomere structure and function in human iPSC-derived cardiac myocytes. *Biochim Biophys Acta*. 2016;1863:1829–1838. doi: 10.1016/j.bbamcr.2015.11.005
62. Lemp NA, Hiraoka K, Kasahara N, Logg CR. Cryptic transcripts from a ubiquitous plasmid origin of replication confound tests for cis-regulatory function. *Nucleic Acids Res*. 2012;40:7280–7290. doi: 10.1093/nar/gks451
63. Juven-Gershon T, Cheng S, Kadonaga JT. Rational design of a super core promoter that enhances gene expression. *Nat Methods*. 2006;3:917–922. doi: 10.1038/nmeth937
64. Dimas AS, Deutsch S, Stranger BE, Montgomery SB, Borel C, Attar-Cohen H, Ingle C, Beazley C, Gutierrez Arcelus M, Sekowska M, et al. Common regulatory variation impacts gene expression in a cell type-dependent manner. *Science*. 2009;325:1246–1250. doi: 10.1126/science.1174148
65. Dunham I, Kundaje A, Aldred SF, Collins PJ, Davis CA, Doyle F, Epstein CB, Frietze S, Harrow J, Kaul R, et al. An integrated encyclopedia of DNA elements in the human genome. *Nature*. 2012;489:57–74. doi: 10.1038/nature11247
66. Andrey G, Montavon T, Mascrez B, Gonzalez F, Noordermeer D, Leleu M, Trono D, Spitz F, Duboule D. A switch between topological domains underlies HoxD genes collinearity in mouse limbs. *Science*. 2013;340:1234167. doi: 10.1126/science.1234167
67. Osterwalder M, Barozzi I, Tissières V, Fukuda-Yuzawa Y, Mannion BJ, Afzal SY, Lee EA, Zhu Y, Plajzer-Frick I, Pickle CS, et al. Enhancer redundancy provides phenotypic robustness in mammalian development. *Nature*. 2018;554:239–243. doi: 10.1038/nature25461
68. Cannavò E, Khoueiry P, Garfield DA, Geeleher P, Zichner T, Gustafson EH, Ciglar L, Korbel JO, Furlong EE. Shadow enhancers are pervasive features of developmental regulatory networks. *Curr Biol*. 2016;26:38–51. doi: 10.1016/j.cub.2015.11.034
69. Chatterjee S, Kapoor A, Akiyama JA, Auer DR, Lee D, Gabriel S, Berrios C, Pennacchio LA, Chakravarti A. Enhancer variants synergistically drive dysfunction of a gene regulatory network in hirschsprung disease. *Cell*. 2016;167:355–368.e10. doi: 10.1016/j.cell.2016.09.005
70. Stieber J, Herrmann S, Feil S, Löster J, Feil R, Biel M, Hofmann F, Ludwig A. The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. *Proc Natl Acad Sci USA*. 2003;100:15235–15240. doi: 10.1073/pnas.2434235100
71. Duhme N, Schweizer PA, Thomas D, Becker R, Schröter J, Barends TR, Schlichting I, Draguhn A, Bruehl C, Katus HA, et al. Altered HCN4 channel C-linker interaction is associated with familial tachycardia-bradycardia syndrome and atrial fibrillation. *Eur Heart J*. 2013;34:2768–2775. doi: 10.1093/eurheartj/ehs391
72. Milanesi R, Baruscotti M, Gnecchi-Ruscone T, DiFrancesco D. Familial sinus bradycardia associated with a mutation in the cardiac pacemaker channel. *N Engl J Med*. 2006;354:151–157. doi: 10.1056/NEJMoa052475
73. Macri V, Mahida SN, Zhang ML, Sinner MF, Dolmatova EV, Tucker NR, McLellan M, Shea MA, Milan DJ, Lunetta KL, et al. A novel trafficking-defective HCN4 mutation is associated with early-onset atrial fibrillation. *Heart Rhythm*. 2014;11:1055–1062. doi: 10.1016/j.hrthm.2014.03.002
74. Amasyali B, Kilic A, Kilit C. Sinus node dysfunction and atrial fibrillation: which one dominates? *Int J Cardiol*. 2014;175:379–380. doi: 10.1016/j.ijcard.2014.05.043
75. John RM, Kumar S. Sinus node and atrial arrhythmias. *Circulation*. 2016;133:1892–1900. doi: 10.1161/CIRCULATIONAHA.116.018011
76. O'Neal WT, Almahmoud MF, Soliman EZ. Resting heart rate and incident atrial fibrillation in the elderly. *Pacing Clin Electrophysiol*. 2015;38:591–597. doi: 10.1111/pace.12591
77. Villar D, Berthelot C, Aldridge S, Rayner TF, Lukk M, Pignatelli M, Park TJ, Deaville R, Erichsen JT, Jasinska AJ, et al. Enhancer evolution across 20 mammalian species. *Cell*. 2015;160:554–566. doi: 10.1016/j.cell.2015.01.006
78. Ruiz-Villalba A, Mattiotti A, Gunst QD, Cano-Ballesteros S, van den Hoff MJ, Ruijter JM. Reference genes for gene expression studies in the mouse heart. *Sci Rep*. 2017;7:24. doi: 10.1038/s41598-017-00043-9
79. van Tuyn J, Pijnappels DA, de Vries AA, de Vries I, van der Velde-van Dijke I, Knaän-Shanzer S, van der Laarse A, Schalij MJ, Atsma DE. Fibroblasts from human postmyocardial infarction scars acquire properties of cardiomyocytes after transduction with a recombinant myocardin gene. *FASEB J*. 2007;21:3369–3379. doi: 10.1096/fj.07-8211com
80. Claycomb WC, Lanson NA Jr, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ Jr. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci USA*. 1998;95:2979–2984. doi: 10.1073/pnas.95.6.2979
81. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*. 1979;7:1513–1523. doi: 10.1093/nar/7.6.1513
82. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29:15–21. doi: 10.1093/bioinformatics/bts635
83. Koboldt DC, Larson DE, Wilson RK. Using VarScan 2 for germline variant calling and somatic mutation detection. *Curr Protoc Bioinformatics*. 2013;44:15.4.1–15.417. doi: 10.1002/0471250953.bi1504s44
84. Ruijter JM, Van Kampen AH, Baas F. Statistical evaluation of SAGE libraries: consequences for experimental design. *Physiol Genomics*. 2002;11:37–44. doi: 10.1152/physiolgenomics.00042.2002
85. Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Cech M, Chilton J, Clements D, Coraor N, Grüning BA, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res*. 2018;46:W537–W544. doi: 10.1093/nar/gky379
86. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res*. 2009;37:e45. doi: 10.1093/nar/gkp045
87. Verheule S, Sato T, Everett T 4th, Engle SK, Otten D, Rubart-von der Lohe M, Nakajima HO, Nakajima H, Field LJ, Olgin JE. Increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF-beta1. *Circ Res*. 2004;94:1458–1465. doi: 10.1161/01.RES.0000129579.59664.9d
88. Verheule S, van Batenburg CA, Coenjaerts FE, Kirchhoff S, Willecke K, Jongsma HJ. Cardiac conduction abnormalities in mice lacking the gap junction protein connexin40. *J Cardiovasc Electrophysiol*. 1999;10:1380–1389. doi: 10.1111/j.1540-8167.1999.tb00194.x
89. Kolanowski TJ, Antos CL, Guan K. Making human cardiomyocytes up to date: derivation, maturation state and perspectives. *Int J Cardiol*. 2017;241:379–386. doi: 10.1016/j.ijcard.2017.03.099
90. Piccini I, Rao J, Seebohm G, Greber B. Human pluripotent stem cell-derived cardiomyocytes: genome-wide expression profiling of long-term in vitro maturation in comparison to human heart tissue. *Genom Data*. 2015;4:69–72. doi: 10.1016/j.gdata.2015.03.008
91. Churko JM, Garg P, Treutlein B, Venkatasubramanian M, Wu H, Lee J, Wessells QN, Chen SY, Chen WY, Chetal K, et al. Defining human cardiac transcription factor hierarchies using integrated single-cell heterogeneity analysis. *Nat Commun*. 2018;9:4906. doi: 10.1038/s41467-018-07333-4
92. Schiaffino S, Gorza L, Ausoni S. Troponin isoform switching in the developing heart and its functional consequences. *Trends Cardiovasc Med*. 1993;3:12–17. doi: 10.1016/1050-1738(93)90022-X