Integrating Genetic, Transcriptional, and Functional Analyses to Identify 5 Novel Genes for Atrial Fibrillation

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Background—Atrial fibrillation (AF) affects >30 million individuals worldwide and is associated with an increased risk of stroke, heart failure, and death. AF is highly heritable, yet the genetic basis for the arrhythmia remains incompletely understood.

Methods and Results—To identify new AF-related genes, we used a multifaceted approach, combining large-scale genotyping in 2 ethnically distinct populations, cis-eQTL (*expression quantitative trait loci*) mapping, and functional validation. Four novel loci were identified in individuals of European descent near the genes *NEURL* (rs12415501; relative risk [RR]=1.18; 95% confidence interval [CI], 1.13–1.23; $P=6.5\times10^{-16}$), *GJA1* (rs13216675; RR=1.10; 95% CI, 1.06–1.14; $P=2.2\times10^{-8}$), *TBX5* (rs10507248; RR=1.12; 95% CI, 1.08–1.16; $P=5.7\times10^{-11}$), and *CAND2* (rs4642101; RR=1.10; 95% CI, 1.06–1.14; $P=9.8\times10^{-9}$). In Japanese, novel loci were identified near *NEURL* (rs6584555; RR=1.32; 95% CI, 1.26–1.39; $P=2.0\times10^{-25}$) and *CUX2* (rs6490029; RR=1.12; 95% CI, 1.08–1.16; $P=3.9\times10^{-9}$). The top single-nucleotide polymorphisms or their proxies were identified as cis-eQTLs for the genes *CAND2* ($P=2.6\times10^{-19}$), *GJA1* ($P=2.66\times10^{-6}$), and *TBX5* ($P=1.36\times10^{-5}$). Knockdown of the zebrafish orthologs of NEURL and CAND2 resulted in prolongation of the atrial action potential duration (17% and 45%, respectively).

Conclusions—We have identified 5 novel loci for AF. Our results expand the diversity of genetic pathways implicated in AF and provide novel molecular targets for future biological and pharmacological investigation. **(*Circulation*. 2014;130:1225-1235.)**

Key Words: atrial fibrillation ■ epidemiology ■ genetics ■ gene expression ■ polymorphism ■ single nucleotide ■ zebrafish

Received March 19, 2014; accepted August 1, 2014.

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Guest Editor for this article was Kari E. North, PhD.

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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.114.009892/-/DC1.

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Atrial fibrillation (AF) is a common arrhythmia with major public health implications because of its high prevalence, significant morbidity, and considerable associated healthcare costs.[1] Currently, there are nearly 3 million individuals in the United States and >8.8 million individuals in Europe who are affected by AF. With an aging population, the prevalence of AF is expected to increase dramatically. In addition to conventional risk factors,[2] a genetic predisposition has been shown to contribute to AF risk.[3] Over the last several years, numerous AF-associated mutations, candidate genes, and risk loci have been identified; however, much of the heritability of AF remains unexplained.

Clinical Perspective on p 1235

Genome-wide association studies (GWASs) have identified thousands of genetic loci associated with a wide range of conditions and traits. Most studies use a stringent threshold of genome-wide significance that, although minimizing false-positive associations, often fails to identify many disease-associated loci. Increasing the sample size of a GWAS will enhance the power, but for many diseases, large numbers of affected individuals are unavailable, and genotyping remains expensive. Because we have a limited understanding of the pathophysiology of AF, genetic discovery provides an important tool to identify novel pathways and therapeutic targets for the arrhythmia. Given these challenges, we sought to identify AF susceptibility loci using a combination of genotyping, expression quantitative trait loci (eQTL) mapping, and functional validation.

Methods

Overall Study Design

We have used available genome-wide association data sets for AF in Europeans and Japanese and have identified selected genetic variants for additional replication in independent individuals. After separate analyses in each replication cohort, we meta-analyzed the novel findings with the respective prior derivation stages. Variants that reached genome-wide significance for association with AF were subjected to additional analyses. First, we performed eQTL mapping in publicly available domains and left atrial tissue samples to identify gene expression changes depending on the identified genotypes. Second, we applied implicated loci pathway and gene enrichment analyses to better characterize novel candidate genes. Third, we performed candidate gene knockdown in an embryonic zebrafish model to test for morphological and functional changes resulting from gene expression changes. Fourth, we conducted coimmunoprecipitation of candidate genes to inform protein-based interactions of our novel candidate genes. Finally, we looked up our association findings in a large consortial data set of patients with ischemic stroke, a major consequence of AF. The study design, including main results, is summarized in Figure 1.

Study Samples

Potential novel AF susceptibility signals in Europeans and Japanese were selected from a discovery sample consisting of cohorts with incident and prevalent AF, which has been previously described.[4] To replicate variants from the discovery sample, we recruited additional samples and cohorts with available DNA for direct genotyping or existing GWAS data for in silico analysis. European replication samples included 6691 independent AF cases and 17 144 controls. In Japanese, an additional 1618 AF cases and 17 190 controls were analyzed; in a second replication stage, another 5912 AF cases were added, totaling 8373 AF cases. A detailed description of replication cohorts is available in the Methods section in the online-only Data Supplement. Institutional Review boards or Ethics committees approved each contributing site. All participants provided written informed consent for participation in the cohorts, particularly allowing the analysis of DNA for genetic studies.

Selection of Single Nucleotide Polymorphisms for Replication

To identify single nucleotide polymorphisms (SNPs) for replication analyses in Europeans, we used the meta-analysis data set from the GWAS performed by the AFGen consortium[4] and performed several selection steps: First, we selected all SNPs (n=195) that demonstrated suggestive associations with the arrhythmia as defined by a meta-analysis value of $P<5\times10^{-5}$. This significance threshold for SNP inclusion was based on the expected power given an estimated independent validation sample size. Second, we subjected SNPs within 1 Mb of the published genome-wide significant loci to further selection. All SNPs with a linkage disequilibrium measure $r^2\geq0.1$ with the published top signals were omitted to avoid the inclusion of SNPs tagging the published results. Third, we selected all SNPs with a minor allele frequency ≥5%. SNPs with a minor allele frequency <5% were included if they were located in exons or the 3′ untranslated region of known genes. Finally, we selected 49 variants. Given the smaller initial sample size of the GWAS in Japanese, a more extensive list of SNPs was considered for replication on the basis of genotyping platform availability and cost. Balancing our statistical power and genotyping considerations, we thus selected the top 500 SNPs at 350 independent loci from a prior meta-analysis for successive rounds of genotyping as described in the Methods section in the online-only Data Supplement.[4]

Genotyping

Cohorts of European descent were directly genotyped with the iPlex matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technique based on Sequenom platforms. All genotypes were analyzed by use of dedicated calling software applying the manufacturer's recommendations. In Ottawa, TaqMan assays (Applied Biosystems, Inc, Foster City, CA) were used. For in silico replication cohorts, genotypes from commercially available Affymetrix and Illumina genotyping arrays were used. Each cohort used genotyping results imputed to >2.5 million HapMap SNPs based on the HapMap CEPH (Utah residents with ancestry from northern and western Europe) panel. Cohort-specific details are described in Table I in the online-only Data Supplement. For genotyping in Japanese cases, the multiplex polymerase chain reaction–based Invader Assay (Third Wave Technologies) was used according to the manufacturer's recommendations. Quality control for all genotyping results required a call rate ≥99% in both cases and controls, and deviations from the Hardy-Weinberg equilibrium were accepted to a value of $P>1.0\times10^{-6}$ in controls.

Statistical Methods in Europeans

For genetic associations, studies from the GWAS discovery stage were calculated as described earlier.[4] In the replication cohorts, we used logistic regression models to assess the associations between SNPs and AF; to achieve higher statistical power in smaller replication cohorts, we combined prevalent and incident AF cases. All models were adjusted for age at DNA draw and sex. Cohorts with multiple study centers were further adjusted for site. Associations derived from GWAS data sets were also adjusted for principal components to account for population structure. Each cohort contributing in silico replicated SNPs used significant principle components specific to their data set. We assumed an additive model of inheritance. Associations were restricted to SNPs selected according to the description above. Directly genotyped SNPs were used following standard genotyping quality control. For imputed SNPs from GWAS cohorts, we used the observed to expected variance of the imputed SNP genotype count (r^2) to adjudicate the imputation quality, and we included only SNPs with $r^2\geq0.3$ (range, 0–1; 0=random imputation, 1=perfect imputation).

We meta-analyzed study-specific association results using the software Meta-Analysis Helper (METAL), applying a fixed-effects approach weighted for the inverse of variance. Association effects are presented as relative risks (RRs). For significant SNPs, we also computed tests of heterogeneity among the study effects; the P values for

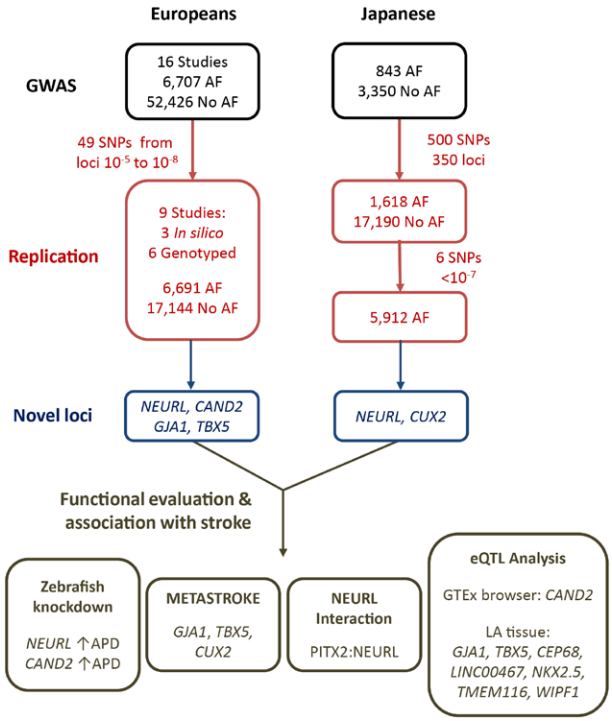

Figure 1. Flow chart illustrating the study design and major results. Novel chromosomal loci associated with atrial fibrillation (AF) were identified independently in cohorts of European and Japanese descent by means of genome-wide association studies (GWAS) and subsequent replication. Signals in or around *NEURL*, *TBX5*, *CAND2*, *GJA1*, and *CUX2* were detected. Additional studies revealed increased atrial action potential durations after knockdown of *NEURL* and *CAND2* in embryonic zebrafish; an interaction between NEURL and PITX2; an association of *GJA1*, *TBX5*, and *CUX2* with stroke; and expression quantitative trait loci (eQTL) associations with *CAND2*, *GJA1*, *TBX5*, *CEP68, LINC00467, NKX2.5, TMEM116,* and *WIPF1* in left atrial (LA) and other tissues. APD indicates action potential duration; and SNP, single nucleotide polymorphism.

the 4 SNPs in Table 1 were all >0.05 and thus not significant. We considered novel loci significantly associated when they exceeded the commonly accepted threshold of genome-wide significance at $P=5\times10^{-8}$ after meta-analyzing our GWAS discovery cohorts with the replication cohorts. For novel loci, we drew regional association plots using LocusZoom considering up to ±1000 kb around the respective top SNP.

Statistical Methods in Japanese

The associations of all SNPs were assessed with the Cochran-Armitage trend test. To further validate the results of the discovery-stage analysis, we selected the 500 SNPs with the most significant Cochran-Armitage trend *P* values for follow-up analyses in an additional 1618 Japanese AF cases and 17 190 AF-free controls. Of the selected 500 SNPs, 150 showed evidence of strong linkage disequilibrium with other selected markers as assessed by the Haploview software. We thus selected 350 SNPs for further genotyping. We combined the genotype data of both the first and second stages for meta-analysis using the Mantel-Haenszel method. We also assessed the heterogeneity of our results for all significantly associated SNPs calculating Breslow-Day tests. All tests yielded values of $P>0.05$ and were thus nonsignificant: rs6584555, $P=0.90$; rs6490029, $P=0.14$; rs639652, $P=0.46$; rs1906599, $P=0.77$; rs6466579, $P=0.27$; and rs12932445, $P=0.98$.

Analysis of eQTLs

We performed eQTL analyses from 2 sources: the Cleveland Clinic Atrial Tissue Bank and the publicly available Genotype-Tissue Expression Portal of the Broad Institute of Harvard and MIT. We first searched for all 49 SNPs considered for replication analysis in Europeans and the 2 SNPs identified in Japanese. Second, for those SNPs exceeding or approaching genome-wide significance after replication (Table 1), we additionally searched for all proxy SNPs, defined as those with at least moderate linkage disequilibrium ($r^2\geq0.5$) with the sentinel SNPs. Detailed methods are provided in the online-only Data Supplement.

Implicated Loci Pathways

We also performed gene enrichment analyses at our implicated loci to determine known functional interactions between the 5 newly discovered loci and the 9 previously reported AF loci,[4] in addition to 6 genes from eQTL analysis. The Web-based tool GRAIL analyzes the connectivity between genetic loci using information retrieved from text mining.[5] Here, we combined 20 loci, including the 14 AF loci and 6 eQTL genes, as both the query and seed regions. The search was performed on abstracts in PubMed published before August 2012. Of the 20 queried loci, 10 showed an excess of connectivity ($P_{GRAIL}<0.05$ after multiple-testing correction). These loci were connected by key words such as cardiac, heart, channels, atrial, or similar. In addition, we used the Ingenuity Pathway Analysis tool to examine functional enrichment of the 14 AF loci. For each locus, we searched genes within 1 Mb of the top SNP. A total of 275 genes were found. These genes were then analyzed by Ingenuity Pathway Analysis, and the most significant canonical pathways were reported.

Knockdown of Candidate Genes in Zebrafish

Zebrafish of the Tübingen/AB strain were maintained according to standard methods. Morpholino oligonucleotides designed to disrupt the proper splicing or translation of zebrafish genes *neurla*, *cand2*, *cand1*, and *cux2b* were obtained from Genetools LLC (Corvallis, OR). Measurements of heart rate and contractile function and optical mapping were obtained as previously described.[6] Details are provided in the online-only Data Supplement.

Coimmunoprecipitation in COS7 Cells

For coimmunoprecipitation in COS7 cells, we transfected an expression plasmid of Myc- or FLAG-tagged target genes into COS7 cells (HSRRB; JCRB9127) using Fugene 6 (Roche). At 24 hours after transfection, immunoprecipitations were performed in lysis buffer (20 mmol/L Tris, pH 7.5, with 150 mmol/L NaCl, 0.4% Nonidet P-40 containing 5 μg/mL MG-132 and protease inhibitor tablet EDTA-Roche) using anti–Myc-tagged (Santa Cruz) or anti–FLAG-tagged M2 agarose (Sigma). We visualized targets using horseradish peroxidase–conjugated anti-FLAG (Sigma) or anti-Myc antibodies (Santa Cruz).

Results

Study Design

The overall design of the study is illustrated in Figure 1. In Europeans, the AFGen discovery sample comprised 16 studies that included 6707 AF cases and 52 426 AF-free controls.[4] There were 195 SNPs with *P* values between 1×10^{-5} and 5×10^{-8} in the AFGen discovery sample. On the basis of a priori power calculations, we then selected 49 SNPs that were not in strong linkage disequilibrium with previously identified loci ($r^2<0.1$). The SNPs were directly genotyped in 6 studies, and in silico replication was performed in 3 studies, together consisting of 6691 independent AF cases and 17 144 controls (Tables I and II in the online-only Data Supplement). The mean age of the AF cases was 64.2±8.3 years compared with 66.1±7.9 years for controls. Approximately two thirds of cases and half of the controls were male.

After meta-analysis of the replication cohorts with the discovery-stage results from the AFGen Consortium, 4 SNPs exceeded the threshold of genome-wide significance in Europeans; 3

Table 1. Meta-analyses of SNP Associations With AF by Origin of Study

SNP	Chromosome	AF Risk Allele	Closest Gene	Relative Location	Original GWAS Data Set[4] RAF	Original GWAS Data Set[4] RR (95% CI)	Original GWAS Data Set[4] P	Replication RAF	Replication RR (95% CI)	Replication P	Overall Meta-Analysis RAF	Overall Meta-Analysis RR (95% CI)	Overall Meta-Analysis P
Europeans													
rs12415501	10q24	T	*NEURL*	Intronic	0.16	1.15 (1.10–1.22)	9.0×10^{-8}	0.16	1.22 (1.14–1.29)	6.0×10^{-10}*	0.16	1.18 (1.13–1.23)	6.5×10^{-16}*
rs10507248	12q24	T	*TBX5*	Intronic	0.73	1.13 (1.08–1.18)	8.5×10^{-8}	0.73	1.11 (1.05–1.17)	0.0001*	0.73	1.12 (1.08–1.16)	5.7×10^{-11}*
rs4642101	3p25	G	*CAND2*	Intronic	0.65	1.11 (1.06–1.15)	4.2×10^{-6}	0.65	1.09 (1.04–1.15)	0.0006*	0.65	1.10 (1.06–1.14)	9.8×10^{-9}*
rs13216675	6q22	T	*GJA1*	Intergenic	0.69	1.10 (1.05–1.15)	5.0×10^{-5}	0.68	1.10 (1.05–1.16)	0.0001*	0.69	1.10 (1.06–1.14)	2.2×10^{-8}*
Japanese													
rs6584555	10q24	C	*NEURL*	Intronic	0.12	1.33 (1.14–1.55)	2.8×10^{-4}	0.12	1.32 (1.25–1.39)	1.6×10^{-22}*	0.12	1.32 (1.26–1.39)	2.0×10^{-25}*
rs6490029	12q24	A	*CUX2*	Intronic	0.65	1.22 (1.09–1.37)	6.3×10^{-4}	0.64	1.11 (1.07–1.16)	5.0×10^{-7}*	0.64	1.12 (1.08–1.16)	3.9×10^{-9}*

In both the discovery and replication stages, each cohort provided cohort-specific results, which were subsequently meta-analyzed. In the overall meta-analysis, the summary results of each stage were meta-analyzed, treating each stage as a cohort. In Ottawa, we used rs3825214 as a proxy SNP for rs12415501 (r^2=0.76). AF indicates atrial fibrillation; CI, confidence interval; GWAS, genome-wide association study; RAF, risk allele frequency; RR, relative risk; and SNP, single-nucleotide polymorphism.

*Significant.

further signals were near genome-wide significance ($P<5\times10^{-8}$). Results for the top 4 variants are shown in Table 1; full results for all 49 SNPs are provided in Tables III and IV in the online-only Data Supplement. Regional association plots for the top 4 associations in Europeans are shown in Figure 2.

In Japanese, the GWAS discovery sample consisted of 843 AF cases and 3350 AF-free controls.[4] A total of 500 SNPs from 350 loci were genotyped in a replication sample consisting of 1618 AF cases and 17 190 controls, and the results were meta-analyzed with the Japanese GWAS discovery data. Six novel SNPs reaching $P<1\times10^{-7}$ were genotyped in 5912 additional AF cases of Japanese ancestry, expanding the total number of AF cases to 8373 (Table V in the online-only Data Supplement); 2 SNPs remained significantly associated with AF (Table 1). Regional association plots for the 2 novel variants in Japanese are shown in Figure 2.

Five Novel AF Risk Loci in Europeans and Japanese

The most significantly associated novel variants in both Europeans and Japanese were intronic to the gene *NEURL* on chromosome 10q24.33 (Europeans: rs12415501; RR for the AF risk allele, 1.18; 95% confidence interval [CI], 1.13–1.23; $P=6.5\times10^{-16}$; Japanese: rs6584555; RR, 1.32; 95% CI, 1.26–1.39; $P=2.0\times10^{-25}$). Fine mapping of 10 additional SNPs at the *NEURL* locus in the Japanese population did not reveal any independent susceptibility signals for AF at this locus (Table VI in the online-only Data Supplement).

The second locus identified in Europeans is intronic to *TBX5* on chromosome 12q24 (rs10507248; RR, 1.12; 95% CI, 1.08–1.16; $P=5.7\times10^{-11}$). The third locus identified in Europeans is on chromosome 3p25.2 intronic to *CAND2* (rs4642101; RR, 1.10; 95% CI, 1.06–1.14; $P=9.8\times10^{-9}$). The SNP rs4642101 is in moderate to strong linkage disequilibrium (r^2=0.64) with the nonsynonymous SNP rs2305398 that results in an amino acid substitution from glutamine to arginine (p.Q315R). The fourth locus identified in Europeans is on chromosome 6q22.31 in a large intergenic region (rs13216675; RR, 1.10; 95% CI, 1.06–1.14; $P=2.2\times10^{-8}$). The closest gene is *GJA1*; rs13216675 is located ≈670 kb downstream of the gene. Interestingly, each of the variants identified in Europeans at *TBX5*, *CAND2*, and *GJA1* was also associated with AF in Japanese (Table VII in the online-only Data Supplement). The fifth locus, which was identified only in Japanese individuals, is located intronic to *CUX2* (rs6490029; RR, 1.12; 95% CI, 1.08–1.16; $P=3.9\times10^{-9}$) on chromosome 12q24.11-12; we did not observe evidence of an association at the *CUX2* locus in Europeans (Figure I and Table VII in the online-only Data Supplement).

eQTL Loci Mapping

We assessed the influence of novel susceptibility signals on the expression of candidate genes by investigating eQTLs using 2 sources. First, accessing the publicly available Genotype-Tissue Expression Portal, we found several significant associations between gene expression and novel susceptibility loci (Table VIII in the online-only Data Supplement). The AF risk allele of the top SNP at the *CAND2* locus, rs4642101, was significantly associated with a higher expression of *CAND2* in skeletal muscle ($P=2.6\times10^{-9}$). A proxy SNP for rs4642101 also had a significant eQTL with *CAND2* (rs9877049; $P=2.6\times10^{-19}$; r^2=0.64). No eQTLs were identified in the Genotype-Tissue Expression Portal database at the 4 other novel loci.

Second, we associated SNP genotypes with gene expression levels in a large repository of left atrial tissue samples (n=289; Table VIII in the online-only Data Supplement). AF was present at the time of tissue acquisition in 136 patients; 70 had no history of AF; and 80 patients were women. Among SNPs at the novel loci for AF, we found significant cis-eQTL associations for which the AF risk allele correlated with a decreased expression of *GJA1* (rs13216675; $P=9.84\times10^{-5}$) and the AF risk allele correlated with an increased expression

of *TBX5* (rs10507248; P=2.14 ×10^{-4}). At both loci, we identified SNPs in linkage disequilibrium with the index SNPs but with statistically stronger effects on gene expression: rs2176990 (r^2=0.54 with rs13216675; P=2.66×10^{-6}; 0.93-fold [0.90–0.95] decreased expression per AF risk allele) and rs1946295 (r^2=0.87 with rs10507248; P=1.36×10^{-5}; 1.12-fold [1.08–1.18] increased expression per AF risk allele).

Among the 49 SNPs initially tested for an association with AF in Europeans, we also observed significant eQTLs for SNPs at 5 other genes. These loci were only marginally associated with AF but exceeded the threshold of significance at P<2.03×10^{-4} for eQTL analyses. The respective loci were found for SNPs in or around the candidate genes *CEP68*, *LINC00467*, *NKX2.5*, *TMEM116*, and *WIPF1*. In more detail, rs2723065 (association with AF, P=7.6×10^{-8}) and, in particular, rs2540950 (r^2=0.93 with rs2723065) were strongly associated with the expression of *CEP68* (P=9.70×10^{-17}). The 4 other SNPs had a weaker association with AF but a significant cis-eQTL association with the candidate genes LINC00467 (rs12733930; P value for association with AF=8.2×10^{-4}; P value for eQTL=1.59×10^{-24}), *NKX2.5* (P for AF=1.0×10^{-6}; P for eQTL=8.78×10^{-6}), *TMEM116* (rs6490029; P for

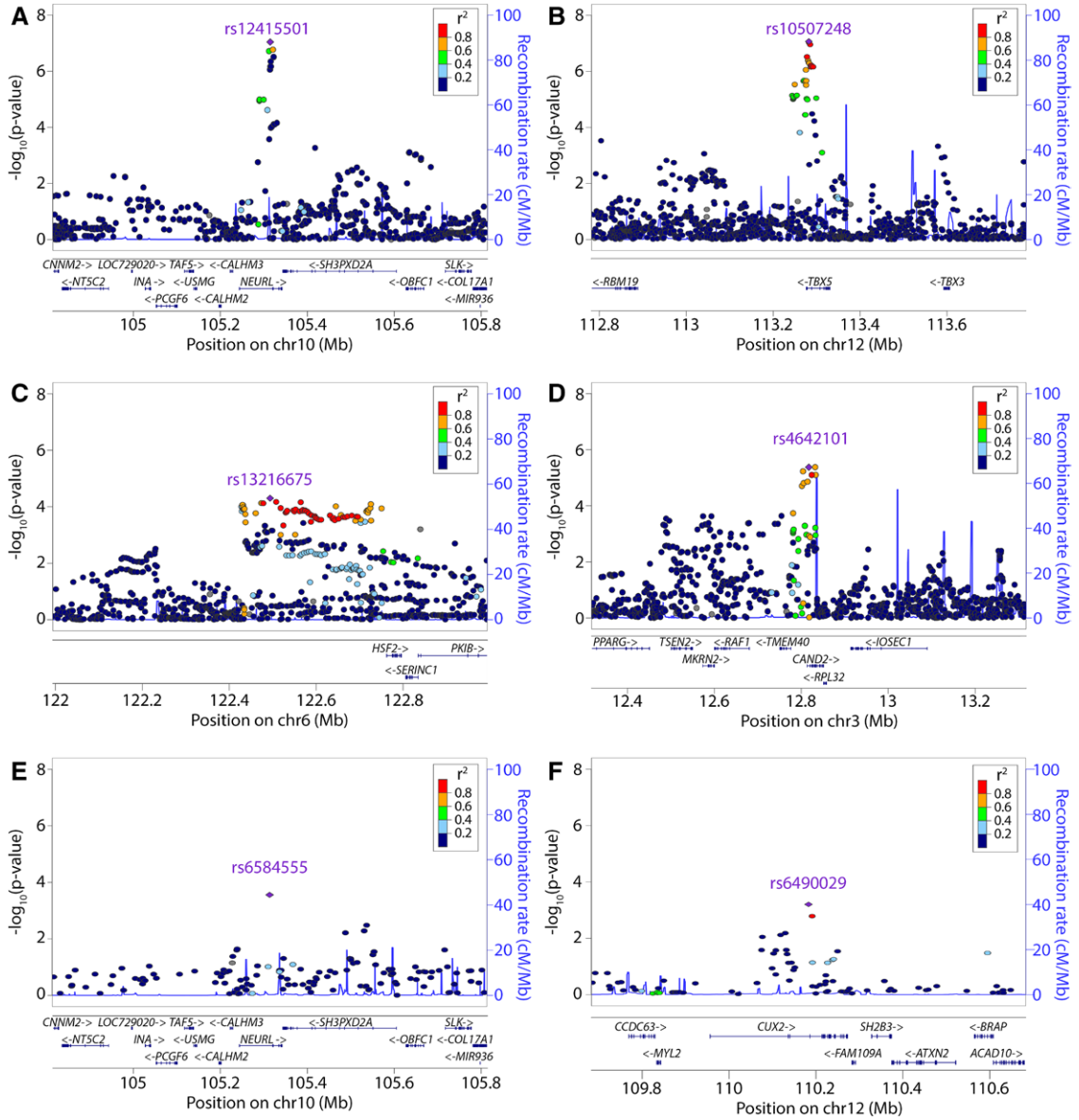

Figure 2. Regional plots for novel atrial fibrillation susceptibility loci in Europeans and Japanese. **A** through **D** (**A**, *NEURL*; **B**, *TBX5*; **C**, *GJA1;* **D**, *CAND2*) show 4 novel loci detected in Europeans; **E** (*NEURL*) and **F** (*CUX2*) show 2 novel loci detected in Japanese. At each novel locus (P≤5×10^{-8}), single nucleotide polymorphisms (SNPs) are plotted using the genomic position (National Center for Biotechnology Information Build 36) and discovery-stage P values. In each panel, the sentinel SNP is labeled in purple. Each dot represents an SNP. The strength of the linkage disequilibrium of SNPs with the sentinel SNP is indicated by a color gradient according to the legend in each panel; red indicates strong and blue indicates weak linkage disequilibrium. Estimated recombination rates are shown by the blue line, and spikes indicate the locations of frequent recombination. Below each panel, the chromosomal positions of the SNPs and regional candidate genes are annotated. Linkage disequilibrium and recombination rates in **A** through **F** are based on the CEPH (Utah residents with ancestry from northern and western Europe) HapMap release 22 (European) and Japanese in Tokyo, Japan (JPT) and Han Chinese in Beijing, China (CHB) JPT+CHB HapMap release 22 (Japanese), respectively. All regional association plots were prepared with the use of LocusZoom.

AF=3.9×10^{-9}; *P* for eQTL=4.28×10^{-6}), and *WIPF1* (rs2358891; *P* for AF=2.0×10^{-6}; *P* for eQTL=8.87×10^{-10}; Table IV in the online-only Data Supplement).

Zebrafish Knockdown Studies of *NEURL*, *CAND2*, and *CUX2*

For the novel AF risk loci identified in our genetic analyses, we sought to determine the potential role of these genes in cardiovascular function through morpholino-mediated knockdown of orthologs in zebrafish embryos (Table IX in the online-only Data Supplement). Because *TBX5* and *GJA1* have well-described roles in cardiovascular physiology, our zebrafish studies focused on the 3 novel candidate genes: *NEURL*, *CAND2,* and *CUX2*.

Zebrafish have a single ortholog of the *NEURL* and *CUX2* genes, *neurla and cux2b,* but have 2 putative orthologs for the *CAND2* gene, *c*and*1* and *c*and*2*. We assessed the efficacy and morphological consequences of gene knockdown and the effect on resting heart rate, ventricular contractility, and atrial action potential duration (APD_{80}). Knockdown efficacy was sufficient for all 4 genes (Table IX in the online-only Data Supplement). Morphologically, embryonic development was only slightly affected by knockdown of *neurla* and *cand1,* which showed mild developmental delay, whereas *cand2* and *cux2b* morphants were indistinguishable from controls (Figure 3A). There were no significant effects on resting ventricular contractile function (Figure 3B) or heart rate (Figure 3C) for any knockdowns. We determined the atrial APD_{80} by analyzing optical mapping data, as described earlier.[6] For *neurla* knockdown embryos, the atrial APD_{80} was significantly lengthened by 17%, 34%, and 19% for the 3 *neurla*-targeting morpholinos (Figure 3D and Table X in the online-only Data Supplement). Knockdown of the zebrafish *cand1* gene resulted in a prolongation of the atrial APD_{80} by 45% (replication morpholino=31% APD_{80} increase) Knockdown of *cand2* or *cux2b* did not significantly alter the APD_{80} (Figure 3D and Table X in the online-only Data Supplement). Representative optical mapping recordings for all 4 gene knockdowns are presented in Figure 3E.

Interaction Between *Neurl* and *Pitx2*

NEURL encodes an E3 ubiquitin ligase with a putative RING finger domain.[7] E3 ubiquitin ligases have been shown to interact with several types of transcription factors.[8] Because a number of AF GWAS loci reside at or near transcription factors (*PITX2*, *ZFHX3*, *PRRX1*, *TBX5*, and *CUX2*), we tested the direct interaction between NEURL and AF-associated transcription factors. NEURL was coexpressed in COS7 cells with each transcription factor using myc- or FLAG-tagged NEURL and myc-tagged PRRX1, ZFHX3, and TBX5 or FLAG-tagged PITX2 and CUX2. Using coimmunoprecipitation, we demonstrated a NEURL-PITX2 protein interaction (Figure IIA in the online-only Data Supplement). We did not find evidence of a direct interaction between NEURL and PRRX1, CUX2, or TBX5; studies on ZFHX3 were unsuccessful (Figure IIB–IID in the online-only Data Supplement).

Implicated Loci Pathways

To integrate our novel SNP and eQTL findings with the previously described 9 susceptibility loci for AF,[4] we used systems biology–based gene enrichment analyses. With the use of the Web-based tool GRAIL, 10 of the total 20 loci showed an excess of connectivity (*P*<0.05), involving key words such as cardiac, heart, channels, and atrial (Figure III in the online-only Data Supplement). The most significantly enriched pathways by an Ingenuity analysis were those involving calcium signaling (*P*=5.3×10^{-5}), L-serine degradation (*P*=4.1×10^{-4}), and geranylgeranyldiphosphate biosynthesis (*P*=8.1×10^{-4}).

Relation Between Novel AF Risk Loci and Stroke

AF is strongly associated with an increased risk of stroke. We therefore determined whether the top 5 novel loci from our genetic analyses were associated with ischemic stroke in the METASTROKE collaboration of the International Stroke Genetics Consortium, a meta-analysis of GWAS combining 12 389 ischemic stroke patients and 62 004 controls (Table 2).[9] For rs6490029, we detected an association with any type of ischemic stroke (*CUX2*; odds ratio, 0.95; 95% CI, 0.91–0.98; *P*=0.0034). Interestingly, the coded allele was hazardous for AF but protective for ischemic stroke. Restricting our analyses to 2365 individuals with cardioembolic stroke, we also found associations for rs13216675 (*GJA1*; odds ratio, 1.11; 95% CI, 1.04–1.19; *P*=0.002) and rs10507248 (*TBX5*; odds ratio, 1.13; 95% CI, 1.05–1.21; *P*=0.0013). Consistent with findings from the METASTROKE collaboration, different subtypes of stroke show limited overlap in genetic associations.[9]

Discussion

In the present study, we sought to integrate multiple parallel techniques to identify novel AF susceptibility loci. Large-scale genotyping in Europeans and Japanese identified novel AF risk loci at or near the genes *NEURL*, *TBX5*, *CAND2*, *GJA1*, and *CUX2*. Expression quantitative trait loci mapping in left atrial tissue identified associations between AF SNPs at the *CAND2*, *TBX5*, *GJA1, CEP68, LINC00467*, *NKX2.5*, *TMEM116*, and *WIPF1* loci. Functional characterization of NEURL and CAND2 orthologs in embryonic zebrafish demonstrated that knockdown of these genes resulted in a significant lengthening of the atrial APD. Furthermore, we found that NEURL and PITX2c physically interacted in a cellular overexpression model. Finally, AF-associated SNPs at the *GJA1*, *TBX5*, and *CUX2* loci were also significantly associated with ischemic stroke.

The most significantly associated novel AF locus that we identified is intronic to the gene *NEURL,* which encodes an E3 ubiquitin ligase. *NEURL* has been reported to be a tumor suppressor gene in malignant astrocytic tumors, and rat and mouse homologs of the gene are highly expressed in muscle tissue.[10] The most consistent cellular abnormalities noted in AF are a calcium overload state and shortening of the atrial APD.[11] Using embryonic zebrafish, we found that knockdown of the NEURL ortholog specifically altered atrial APD without affecting cardiac contractile function or heart rate. Although it is unclear whether the AF-associated SNPs at the locus are associated with an increase or decrease in NEURL expression, our results provide compelling support for the role of NEURL in atrial repolarization and, in turn, AF.[12]

In 2007, a genetic locus was described for AF on chromosome 4q25, upstream from the gene encoding the transcription factor *PITX2;*[13] in the ensuing years, the association between AF and variants at this locus has been widely replicated. Although the role of *PITX2* in AF has not yet been fully understood, it is critical for the left-right symmetry of the heart during embryogenesis and the formation of myocardial

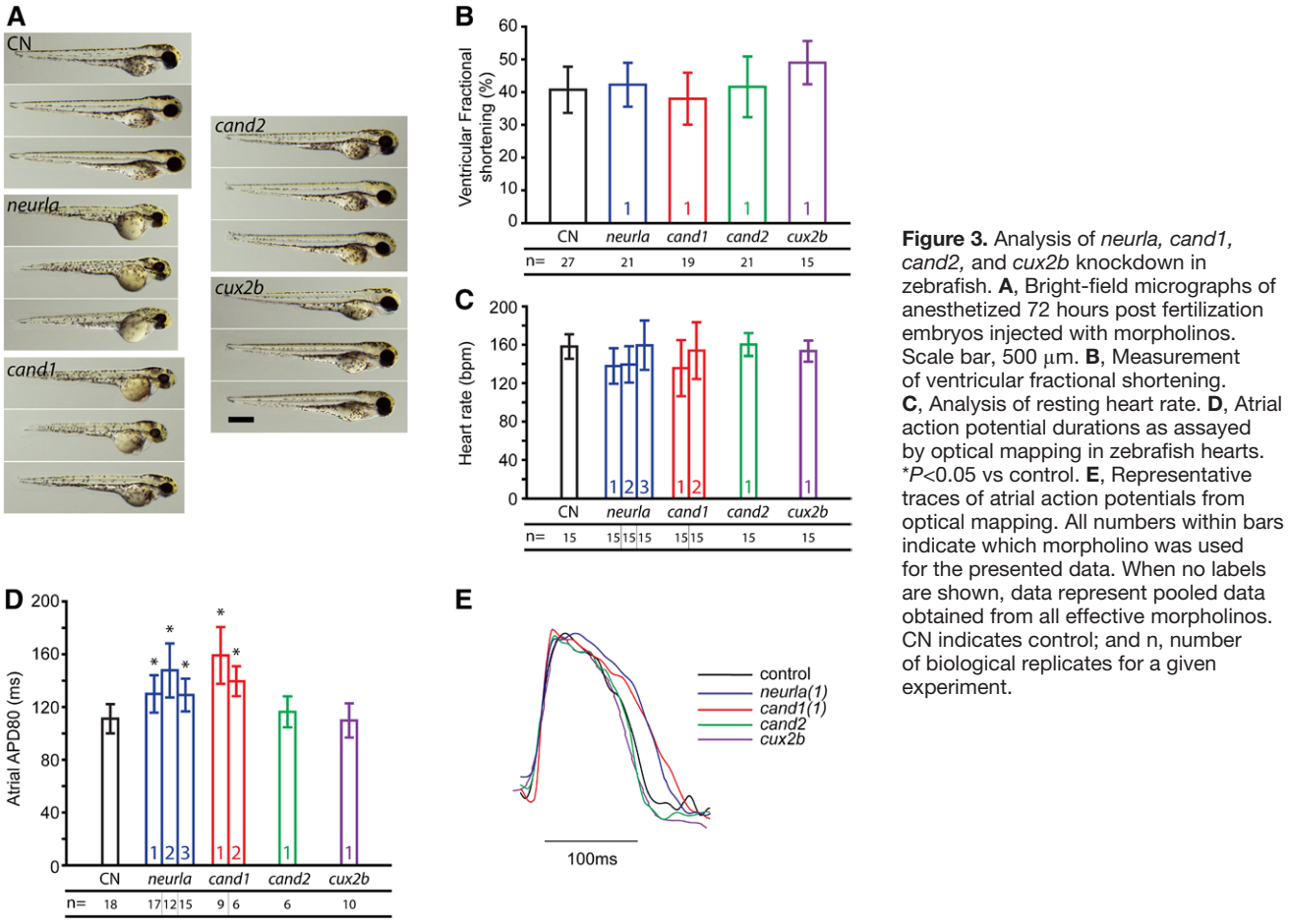

Figure 3. Analysis of *neurla, cand1, cand2,* and *cux2b* knockdown in zebrafish. **A**, Bright-field micrographs of anesthetized 72 hours post fertilization embryos injected with morpholinos. Scale bar, 500 μm. **B**, Measurement of ventricular fractional shortening. **C**, Analysis of resting heart rate. **D**, Atrial action potential durations as assayed by optical mapping in zebrafish hearts. **P*<0.05 vs control. **E**, Representative traces of atrial action potentials from optical mapping. All numbers within bars indicate which morpholino was used for the presented data. When no labels are shown, data represent pooled data obtained from all effective morpholinos. CN indicates control; and n, number of biological replicates for a given experiment.

sleeves in the pulmonary veins.[14] Furthermore, loss of one isoform, PITX2c, has been associated with an increased susceptibility to AF in murine models. Given the in vitro interaction between NEURL and PITX2 that we observed, it is interesting to speculate that NEURL may mediate a susceptibility to AF by ubiquitin-mediated alteration of PITX2 activity.

The second novel locus we identified resides at *TBX5,* a transcription factor that is critically involved in the development of the cardiac conduction system.[15] We also found that SNPs at this locus modulate the expression of *TBX5* in human atrial tissue. Mutations in *TBX5* underlie Holt-Oram syndrome, features of which include atrial and ventricular septum secundum defects and conduction abnormalities, including atrioventricular node block. In an atypical form of Holt-Oram syndrome with a high prevalence of AF, a *TBX5* gain-of-function mutation was identified, a finding that is consistent with our eQTL results.[16] Two recent GWASs associated the ECG PR interval with variants intronic to or in proximity with *TBX5*.[17,18] In the study by Holm et al,[17] the top SNP (rs3825214; r^2=0.76 with rs10507248) also showed association with AF (P=4.0×10^{-5}) but failed to reach genome-wide significance. In the study by Pfeufer et al,[18] rs1896312 was independent of rs10507248 (r^2=0) and showed no association with AF (P=0.72). Interestingly, we also found that expression levels of *NKX2.5* vary by SNP genotypes in our data set. Together, *TBX5* and *NKX2.5* are known to play critical roles in both the differentiation of cardiomyocytes and the specialization of conduction and nodal tissue.[15]

At the third novel locus, *CAND2* encodes a TATA-binding protein, TIP120b, which is muscle specific and critical for myogenesis.[19] We found that the AF-associated SNP at this locus is associated with reduced CAND2/TIP120b expression in striated muscle tissue. Although the specific role of CAND2/TIP120b in AF is currently unclear, we observed atrial action potential prolongation by morpholino-mediated gene knockdown in zebrafish. Additionally, our eQTL analyses indicate that the risk allele is associated with increased expression of CAND2. Extrapolating our findings in zebrafish, increased CAND2 levels would be predicted to shorten the atrial APD, as has been widely observed in AF.

GJA1, a strong candidate gene at our fourth AF locus, encodes the gap junction protein connexin43 on chromosome 6q22.31 which is abundantly expressed in the heart.[20] We found that AF-associated SNPs influenced the transcription of *GJA1* in both left atrial tissue and the whole heart. Connexin43, the predominant cardiac gap junction protein, facilitates coordinated electric activity between adjacent myocytes. Germline mutations in *GJA1* have been associated with syndromic diseases such as hypoplastic left heart syndrome, atrioventricular canal defects, or oculodentodigital dysplasia. Interestingly, a somatic, loss-of-function mutation in connexin43 has been found to underlie AF in humans.[21] Furthermore, mice with 60% reduced atrial *Gja1* expression showed an increased susceptibility to induced AF and atrial tachycardia.[22] Two independent swine models with an AF-induced reduction in *GJA1* expression demonstrated that

Table 2. Association of Novel AF Loci in the METASTROKE Consortium[9]

Phenotype	Cases, n	Controls, n	SNP	AF Risk Allele	Closest Gene	RAF	OR (95% CI)	*P*
Overall ischemic stroke	12 389	62 004	rs12415501	T	*NEURL*	0.16	1.03 (0.99–1.08)	0.20
			rs10507248	T	*TBX5*	0.73	1.05 (1.01–1.08)	0.01
			rs4642101	G	*CAND2*	0.65	0.95 (0.93–0.99)	0.95
			rs13216675	T	*GJA1*	0.69	1.04 (1.01–1.08)	0.02
			rs6490029	A	*CUX2*	0.23	0.95 (0.91–0.98)	0.0034*
Cardioembolic stroke	2365	56 140	rs12415501	T	*NEURL*	0.16	1.10 (1.01–1.20)	0.03
			rs10507248	T	*TBX5*	0.73	1.11 (1.04–1.19)	0.0027*
			rs4642101	G	*CAND2*	0.65	1.04 (0.97–1.11)	0.23
			rs13216675	T	*GJA1*	0.69	1.13 (1.05–1.21)	0.0013*
			rs6490029	A	*CUX2*	0.23	0.98 (0.91–1.05)	0.55

AF indicates atrial fibrillation; CI, confidence interval; RAF, risk allele frequency; RR, relative risk; and SNP, single-nucleotide polymorphism.
*Significant.

restoration of *GJA1* expression ameliorated AF burden.[13] More recently, SNPs in proximity of *GJA1* have been reported to be associated with resting heart rate[23]; however, the AF variants appear to be unrelated to both (r^2=0.02 for each).

At the fifth locus, *CUX2*, cut-like homeobox 2, is a transcription factor implicated in cell-cycle progression relevant for spinal cord development[24] and has been investigated for its contribution to bipolar disorder. More recently, the Wellcome Trust Case Control Consortium identified variants at *CUX2* as a significant susceptibility marker for type 1 diabetes mellitus.[25] Yet, the reported SNP rs1265564 displays only weak linkage disequilibrium (r^2=0.17) with the AF SNP rs6490029. In another GWAS of Koreans and Japanese for coronary artery disease, *CUX2* was suggested as a susceptibility locus but failed to replicate.[26] The *CUX2* association was Japanese specific; we did not find evidence for an association in the region among Europeans (Figure I in the online-only Data Supplement). The specificity of the *CUX2* association in Japanese was in contrast to the other 4 loci that were all associated with AF to various degrees (Table 1 and Table VII in the online-only Data Supplement). The variability in the association between individuals of European and Japanese ancestry may be due to differences in allele frequency or sample size or to another intrinsic difference between the populations.

Clinically, AF confers a 5-fold increased risk of stroke. We found that the AF SNPs at the *CUX2*, *GJA1*, and *TBX5* loci were associated with ischemic stroke in the METASTROKE collaboration. Interestingly, we found that the AF risk allele at the *CUX2* locus was associated with a decreased risk of ischemic stroke, whereas the AF risk alleles at the 2 other loci conferred an increased risk of cardioembolic stroke. Given that 2 of the strongest associations for stroke are at the *PITX2* and *ZFHX3* loci for AF,[27,28] it is possible that the associations we observed at the *GJA1* and *TBX5* loci are due to occult AF among the stroke cases. At present, it remains unclear why variants at *CUX2* would be associated with a decreased risk of ischemic stroke.

Strengths of our work include the investigation of 2 large samples of AF cases in Europeans and Japanese, eQTL analyses in atrial tissue, functional studies supporting the role for *NEURL* and *CAND2* in AF pathophysiology, and the association of 3 of the novel AF loci with stroke. However, our study was also subject to a number of limitations. We studied individuals of European and Japanese ancestry; thus, extrapolation of our findings to other races and ethnicities may be limited. Although AF often occurs in association with other risk factors, we included all individuals with AF to increase both the generalizability and the statistical power of the present analyses. We acknowledge that the NEURL:PITX2 interaction that we observed was in vitro and that further in vivo studies will be necessary. As with other GWASs, the AF-associated SNPs are unlikely to be the causal variants; rather, they are likely to be a marker of disease risk. Although we believe that our eQTL, coimmunoprecipitation, and zebrafish studies were important initial analyses, ultimately, further fine mapping, sequencing, and functional studies will be required to identify the specific role of these genes in the pathogenesis of AF.

Conclusions

Using a combination of genetic associations, eQTL analyses, and functional mapping of novel genes, we have identified 5 susceptibility loci for AF. Functional analyses of NEURL and CAND2 via zebrafish knockdown resulted in alterations in atrial electrophysiology, and protein interaction analysis demonstrated an in vitro interaction between NEURL and PITX2. Finally, our findings indicate that the novel AF signals at *GJA1*, *TBX5,* and *CUX2* were significantly associated with ischemic stroke or its subtypes. In aggregate, our studies expand our understanding of the molecular pathways and clinical implications of this common and morbid arrhythmia.

Appendix

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Sources of Funding

This work was funded by the following: AGES: National Institutes of Health (NIH) contract N01-AG-12100, NIA Intramural Research Program, Hjartavernd (the Icelandic Heart Association), and the Althingi (the Icelandic Parliament). AFNET/KORA S4: German National Genome Research Network NGFN 01GS0838, 01GR0803, BMBF-01EZ0874, 01GR0803, NGFN 01GI0204, 01GR0103, NGFN-2, NGFNPlus 01GS0823, and NGFNPlus 01GS0834; German Federal Ministry of Education and Research (BMBF) 01EZ0874; German Competence Network on AF (AFNET) 01 GI 0204/N; Leducq Foundation 07-CVD 03, BMBF Spitzen cluster personalized medicine m4 (01 EX1021E), LMU Excellence Initiative (42595-6); Munich Center of Health Sciences (MC Health) as part of LMUinnovativ. The KORA platform is funded by the BMBF and by the State of Bavaria. Dr Sinner was supported by the German Heart Foundation. Dr Kääb was supported by the DZHK (German Center for Cardiovascular Research) and BMBF (German Ministry of Education and Research). ARIC: National Heart, Lung, and Blood Institute (NHLBI) contracts N01-HC-55015, N01-HC-55016, N01-HC-55018, N01- HC-55019, N01-HC-55020, N01-HC-55021, N01-HC-55022, R01HL087641, R01HL59367, R01HL086694 and U10HL054512; National Human Genome Research Institute contract U01HG004402; and NIH contract HHSN268200625226C. We thank the staff and participants of the ARIC study for their important contributions. Infrastructure was partly supported by grant UL1RR025005, a component of the NIH and NIH Roadmap for Medical Research. Additional support was provided by grants RC1-HL-099452 from the NHLBI and 09SDG2280087 from the American Heart Association. BioBank Japan: This study was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan and by SENSIN Medical Research Foundation, Japan. DANFIB: Danish National Research Foundation Center for Cardiac Arrhythmia, the John and Birthe Meyer Foundation, the Research Foundation of the Heart Center Rigshospitalet, Lægernes Forsikringsforening af 1891, the Arvid Nilsson Foundation, and the Director Henrik Ibsens Foundation, the Villadsen Family Foundation, the stockbroker Henry Hansen and wife Karla Hansen, born Westergaard, Grant, and the Danish Heart Foundation (grant 07-10-R60-A1815-B573-22398). Cleveland Clinic: R01 HL090620 and R01 HL111314 from the NHLBI (Drs Chung, Barnard, J. Smith, and Van Wagoner); NIH/National Center for Research Resources Clinical and Translational Scienece Award 1UL-RR024989 (Drs Chung and Van Wagoner); Heart and Vascular Institute, Department of Cardiovascular Medicine, Cleveland Clinic (Dr Chung); Leducq Foundation 07-CVD 03 (Drs Van Wagoner and Chung); Atrial Fibrillation Innovation Center, State of Ohio (Drs Van Wagoner and Chung). CHS: NHLBI contracts HHSN268201200036C, HHSN268200800007C, N01HC55222, N01HC85079, N01HC85080, N01HC85081, N01HC85082, N01HC85083, and N01HC85086, and NHLBI grants HL080295, HL087652, and HL105756 with additional contribution from the National Institute of Neurological Disorders and Stroke. Additional support was provided through AG023629 from the National Institute on Aging (NIA). A full list of CHS investigators and institutions can be found at http://www.CHS-NHLBI.org. The provision of genotyping data was supported in part by the National Center for Advancing Translational Sciences, Clinical & Translational Science Institute grant UL1TR000124, and the National Institute of Diabetes and Digestive and

Kidney Disease Diabetes Research Center grant DK063491 to the Southern California Diabetes Endocrinology Research Center. FHS: NHLBI and Boston University School of Medicine based on analyses by Framingham Heart Study investigators participating in the SNP Health Association Resource (SHARe) project. NHLBI's Framingham Heart Study (contract No. N01-HC-25195) and its contract with Affymetrix, Inc for genotyping services (contract No.N02-HL-6-4278). A portion of this research used the Linux Cluster for Genetic Analysis (LinGA-II) funded by the Robert Dawson Evans Endowment of the Department of Medicine at the Boston University School of Medicine and Boston Medical Center. Other support came from 1R01 HL092577 1RO1 HL076784, 1R01 AG028321, Evans Center for Interdisciplinary Biomedical Research ARC on Atrial Fibrillation at Boston University (Benjamin), 6R01-NS 17950, and American Heart Association 09FTF2190028. Health ABC: NIA contracts N01AG62101, N01AG62103, and N01AG62106. The genome-wide association study was funded by NIA grant 1R01AG032098-01A1 to Wake Forest University Health Sciences, and genotyping services were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract from the NIH to The Johns Hopkins University, contract No. HHSN268200782096C. This research was supported in part by the Intramural Research Program of the NIH, NIA. HVH: NHLBI R01 HL 068986, R01 HL085251, and R01 HL073410. MAC: The Malmö Diet and Cancer study was made possible by grants from the Malmö city council. Dr J.G. Smith was supported by the Swedish Heart-Lung foundation and the Thorsten Westerström Foundation. Dr Melander was supported by the Swedish Heart-Lung Foundation, the Swedish Medical Research Council, the Medical Faculty of Lund University, the Malmö University Hospital, the Albert Påhlsson Research Foundation, the Crafoord Foundation, the Region Skane, the Hulda and Conrad Mossfelt Foundation, the King Gustaf V and Queen Victoria Fund, the Lennart Hanssons Memorial Fund, and the Wallenberg Foundation. MGH: Doris Duke Charitable Foundation Clinical Scientist Development Award 2014105 (Dr Lubitz), NIH grants K23HL114724 (Dr Lubitz), T32HL007208 (Dr Tucker), R01HL092577 (Drs Ellinor and Benjamin), HL065962, R01HL104156 and K24HL105780 (Dr Ellinor), and an American Heart Association Established Investigator Award 13EIA14220013 (Ellinor). Ottawa: Heart and Stroke Foundations of Ontario (Dr Gollob), and the Canadian Institutes for Health Research (Dr Gollob). Dr Gollob is supported by the Early Researcher Award program from the government of Ontario and from the Heart and Stroke Foundation of Ontario Clinician-Scientist Award. PROSPER: Supported by an investigator-initiated grant obtained from Bristol-Myers Squibb. Dr Jukema is an Established Clinical Investigator of the Netherlands Heart Foundation (grant 2001 D 032). Support for genotyping was provided by the Seventh Framework Program of the European Commission (grant 223004) and by the Netherlands Genomics Initiative (Netherlands Consortium for Healthy Aging grant 050-060-810). RS-I / RS-II: RS-I is supported by the Erasmus Medical Center and Erasmus University Rotterdam; the Netherlands Organization for Scientific Research; The Netherlands Organization for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly; The Netherlands Heart Foundation; the Ministry of Education, Culture and Science; the Ministry of Health Welfare and Sports; the European Commission; and the Municipality of Rotterdam. Support for genotyping was provided by the Netherlands Organization for Scientific Research (175.010.2005.011, 911.03.012) and Research Institute for Diseases in the Elderly. This study was supported by the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research project No. 050-060-810. SHIP: SHIP is part of the Community Medicine Research Network of the University of Greifswald, Germany, which is funded by the Federal Ministry of Education and Research (grants 01ZZ9603, 01ZZ0103, and 01ZZ0403), the Ministry of Cultural Affairs, and the Social Ministry of the Federal State of Mecklenburg-West Pomerania. This work supported partly by the research project Greifswald Approach to Individualized Medicine, which is funded by the BMBF and the Ministry of Cultural Affairs of the Federal State of Mecklenburg-West Pomerania (03IS2061A). Generation of genome-wide data has been supported by the BMBF (grant 03ZIK012) and a joint grant from Siemens Healthcare, Erlangen, Germany, and the Federal State of Mecklenburg-West Pomerania. The University of Greifswald is a member of the Center of Knowledge Interchange program of the Siemens AG. WGHS: NHLBI HL 043851 (Buring) and HL69757 (Buring) and National Cancer Institute CA 047988 (Buring), the Donald W. Reynolds Foundation and the Fondation Leducq (Ridker), with collaborative scientific support and funding for genotyping provided by Amgen. AF end-point confirmation was supported by HL-093613 (Dr Albert) and a grant from the Harris Family Foundation (Tedrow). METASTROKE: Dr Dichgans is supported by the Vascular Dementia Research Foundation. Dr Rosand is supported by the National Institute of Neurological Disorders and Stroke U01-NS069208. None of the funding agencies had any role in the study design, data collection or analysis, interpretation of the data, writing of the manuscript, or the decision to submit the manuscript for publication.

Disclosures

None.

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CLINICAL PERSPECTIVE

Atrial fibrillation (AF) is a multifactorial disease with many risk factors, including family history. To date, genome-wide association studies have identified 9 susceptibility loci that do not fully explain the heritability of the arrhythmia. In the present work, 5 novel genetic loci for AF were identified in large populations of European and Japanese descent in or near the genes *NEURL*, *TBX5*, *CAND2*, *GJA1*, and *CUX2*. Profiling the expression of candidate genes at these loci, we found that the AF risk variants significantly modified the expressions of *CAND2* in skeletal muscle and of *GJA1* and *TBX5* in left atrial tissue. For *NEURL* and *CAND2*, gene knockdown in a zebrafish model demonstrated a significant prolongation of the atrial action potential duration. Finally, we found that variants at *GJA1*, *TBX5*, and *CUX2* were significantly associated with ischemic stroke. In summary, the present results expand on the pathophysiological mechanisms underlying the development and maintenance of AF. Ultimately, therapeutic approaches that target these pathways may help to reduce the burden of AF and the sequela of heart failure and stroke.