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

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Abstract

Background—Atrial fibrillation (AF) affects over 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 & Results—To identify new AF-related genes, we utilized a multifaceted approach, combining large-scale genotyping in two ethnically distinct populations, cis-eQTL mapping, and functional validation. Four novel loci were identified in individuals of European descent near the genes *NEURL* (rs12415501, RR=1.18, 95%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 SNPs 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* ($p=1.36\times10^{-05}$). 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 five novel loci for AF. Our results further expand the diversity of genetic pathways implicated in AF and provide novel molecular targets for future biological and pharmacological investigation.

Keywords

atrial fibrillation; genetics; epidemiology; expression; functional analysis; zebrafish

Introduction

Atrial fibrillation (AF) is a common arrhythmia with major public health implications due to its high prevalence, significant morbidity and considerable associated healthcare costs.[1] Currently, there are nearly 3 million individuals in the United States and over 8.8 million individuals in Europe affected by AF. With an aging population, the prevalence of AF is expected to dramatically increase. In addition to conventional risk factors,[2] a genetic predisposition has been shown to contribute to AF risk.[3] Over the last several years,

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numerous AF associated mutations, candidate genes, and risk loci have been identified; however, much of the heritability of AF remains unexplained.

Genome wide association studies (GWAS) have identified thousands of genetic loci associated with a wide range of conditions and traits. Most studies employ a stringent threshold of genome wide significance which, while minimizing false-positive associations, often fails to identify many disease associated loci. Increasing the sample size of a GWAS will enhance power, but for many diseases large numbers of affected individuals are unavailable and genotyping remains expensive. Since 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, eQTL mapping, and functional validation.

Methods

Overall study design

We have used available genome-wide association datasets for AF in Europeans and Japanese, respectively, and have identified selected genetic variants for additional replication in independent individuals. Following 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 morphologic and functional changes due to gene expression changes. Fourth, we conducted co-immunoprecipitation of candidate genes to inform protein-based interactions of our novel candidate genes. Last, we looked-up our association findings in a large consortial dataset 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 6,691 independent AF cases and 17,144 controls. In Japanese, an additional 1,618 AF cases and 17,190 controls were analyzed; in a second replication stage, another 5,912 AF cases were added, totaling 8,373 AF cases. A detailed description of replication cohorts is available in the Supplemental Methods. 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 SNPs for replication

To identify SNPs for replication analyses in Europeans, we used the meta-analysis dataset from the GWAS performed by the AFGen consortium,[4] and performed several selection steps: (1) We selected all SNPs (n=195) that demonstrated suggestive associations with the arrhythmia as defined by a meta-analysis p-value $<5\times10^{-5}$. This significance threshold for SNP inclusion was based on the expected power given an estimated independent validation sample size. (2) We then 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. (3) Finally we selected all SNPs with a minor allele frequency (MAF) ≥5%. SNPs with a MAF <5% were included if they were located in exons or the 3' untranslated region (3'UTR) 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 based on 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 supplemental methods.[4]

Genotyping

Cohorts of European descent were directly genotyped using the iPlex matrix assisted laser desorption / ionization time-of-flight (MALDI-TOF) mass spectrometry technique based on Sequenom platforms. All genotypes were analyzed using 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 CEU panel. Cohort-specific details are described in Supplemental Table 1. For genotyping in Japanese cases, the multiplex PCR-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 p-value $>1.0\times10^{-6}$ 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 further adjusted for site. Associations derived from GWAS datasets were also adjusted for principle components to account for population structure. Each cohort contributing *in-silico* replicated SNPs used significant principle components specific to their dataset. 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 only included SNPs with $r^2 \geq 0.3$ (range 0–1; 0: random imputation; 1: perfect imputation).

We meta-analyzed study-specific association results using METAL, applying a fixed effects approach weighted for the inverse of variance. Association effects are presented as relative risks (RR). For significant SNPs, we also computed tests of heterogeneity among the study effects; the p-values for 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 ±1000kb around the respective topSNP.

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 additional 1,618 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 stage for meta-analysis using the Mantel-Haenszel method. We also assessed heterogeneity of our results for all significantly associated SNPs calculating Breslow-Day tests. All tests yielded p-values >0.05 and were thus non-significant: rs6584555, p=0.90; rs6490029, p=014; rs639652, p=0.46; rs1906599, p=0.77; rs6466579, p=0.27; rs12932445, p=0.98.

Analysis of eQTLs

We performed eQTL analyses from two sources: the Cleveland Clinic Atrial Tissue Bank and the publicly available Genotype-Tissue Expression Portal (GTEx) of the Broad Institute of Harvard and MIT. We first searched for all 49 SNPs considered for replication analysis in Europeans as well as 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 (r2≥0.5) with the sentinel SNPs. Detailed methods are provided in the 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 the abstracts in PubMed published before August 2012. Out of the 20 queried loci, 10 showed an excess of connectivity ($P_{GRAIL}<0.05$ after multiple testing correction). These loci were connected by keywords such as "cardiac", "heart", "channels", "atrial", or similar. In addition, we used the Ingenuity Pathway Analysis

(IPA) tool to examine functional enrichment of the 14 AF loci. For each locus, we searched genes within 1Mb of the top SNP. A total of 275 genes were found. These genes were then analyzed by IPA, 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 (MOs) designed to disrupt the proper splicing or translation of zebrafish genes *neurla, cand2, cand1*, and *cux2b* were obtained from Genetools LLC (Corvallis, OR, USA). Measurements of heart rate, contractile function and optical mapping were obtained as previously described;[6] detailed methods are provided in the supplement.

Co-immunoprecipitation in COS7 cells

For co-immunoprecipitation 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 24h post transfection, immunoprecipitations were performed in lysis buffer (20mM Tris pH 7.5, with 150mM NaCl, 0.4% Nonidet P-40 containing 5μg/ml of 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 HRP-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 6,707 AF cases and 52,426 AF free controls.[4] There were 195 single nucleotide polymorphisms (SNPs) with p values between 1×10^{-5} and 5×10^{-8} in the AFGen discovery sample. Based on *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 6,691 independent AF cases and 17,144 controls (Supplemental Tables 1 & 2). The mean age in the AF cases was 64.2±8.3 years versus 66.1±7.9 years in controls. Approximately two thirds of cases and half of the controls were male.

Following meta-analysis of the replication cohorts with the discovery stage results from the AFGen Consortium, four SNPs exceeded the threshold of genome-wide significance in Europeans; three 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 Supplemental Tables 3 & 4. Regional association plots for the top four associations in Europeans are shown in Figure 2.

In Japanese, the GWAS discovery sample consisted of 843 AF cases and 3,350 AF free controls.[4] A total of 500 SNPs from 350 loci were genotyped in a replication sample consisting of 1,618 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

5,912 additional AF cases of Japanese ancestry, expanding the total number of AF cases to 8,373 (Supplemental Table 5); two SNPs remained significantly associated with AF (Table 1). Regional association plots for the two 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, relative risk for the AF risk allele (RR) 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 ten additional SNPs at the *NEURL* locus in the Japanese population did not reveal any independent susceptibility signals for AF at this locus (Supplemental Table 6).

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 non-synonymous 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 approximately 670kb downstream of the gene. Interestingly, each of the variants identified in Europeans at the *TBX5*, *CAND2*, and *GJA1*, were also associated with AF in Japanese (Supplemental Table 7). 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 (Supplemental Figure 1 and Supplemental Table 7).

Expression quantitative trait loci mapping

We assessed the influence of novel susceptibility signals on the expression of candidate genes by investigating eQTLs using two sources. First, accessing the publicly available Genotype-Tissue Expression Portal (GTEx), we found several significant associations between gene expression and novel susceptibility loci (Supplemental Table 8). 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 ($p=2.6\times 10^{-9}$). 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 GTEx database at the four other novel loci.

Second, we associated SNP genotypes with gene expression levels in a large repository of left atrial tissue samples (n=289; Supplemental Table 8). 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 where 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, $p=2.14\times10^{-4}$). 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 five 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 four 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 AF=3.9×10^{-9}, p for eQTL=4.28×10^{-06}), and *WIPF1* (rs2358891, p for AF=2.0×10^{-6}, p for eQTL=8.87×10^{-10}) (Supplemental Table 4).

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 orthologues in zebrafish embryos (Supplemental Table 9). Since *TBX5* and *GJA1* have well-described roles in cardiovascular physiology, our zebrafish studies focused on the three novel candidate genes: *NEURL, CAND2*, and *CUX2*.

Zebrafish have a single ortholog of the *NEURL and CUX2* genes, *neurla and cux2b*, but have two putative orthologs for the *CAND2* gene, *cand1* and *cand2*. We assessed the efficacy and morphologic 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 four genes (Supplemental Table 9). 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 three *neurla*-targeting morpholinos (Figure 3D, Supplemental Table 10). 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, Supplemental Table 10). Representative optical mapping recordings for all four 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] Since 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 co-expressed 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. By co-immunoprecipitation, we demonstrated a NEURL-PITX2 protein interaction (Supplemental Figure 2a). We did not find evidence of a direct interaction between NEURL and PRRX1, CUX2, or TBX5; studies on ZFHX3 were unsuccessful (Supplemental Figure 2b, 2c, 2d).

Implicated loci pathways

To integrate our novel SNP and eQTL findings with the previously described 9 susceptibility loci for AF,[4] we employed systems biology based gene enrichment analyses. Using the web-based tool GRAIL, 10 of the total 20 loci showed an excess of connectivity ($p<0.05$) involving keywords such as "cardiac", "heart", "channels", and "atrial" (Supplemental Figure 3). The most significantly enriched pathways by an Ingenuity analysis were those involving “calcium signaling” ($p=5.3\times10^{-5}$), “L-serine degradation” ($p=4.1\times10^{-4}$), and “geranylgeranyldiphosphate biosynthesis” ($p=8.1\times10^{-4}$).

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 analyses 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 action potential duration. Further, 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 action potential duration.[11] Using embryonic zebrafish, we found that knockdown of the NEURL ortholog specifically altered atrial action potential duration without affecting cardiac contractile function or heart rate. While 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*;[22] 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 sleeves in the pulmonary veins.[13] Further, 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.[14] 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, findings that are consistent with our eQTL results.[15] Two recent GWAS associated the electrocardiographic PR interval with variants intronic to or in proximity with *TBX5*.[16, 17] In the study by Holm et al., the top SNP (rs3825214, r^2=0.76 with rs10507248) also showed association with AF ($p=4.0\times10^{-5}$), but failed to reach genome-wide significance.[16] In the study by Pfeufer et al., rs1896312 is independent of rs10507248 (r^2=0) and showed no association with AF (p=0.72).[17] Interestingly, we also found expression levels of *NKX2.5* vary by SNP genotypes in our dataset. 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.[14]

At the third novel locus, *CAND2* encodes a TATA-binding protein, TIP120b, which is muscle-specific and critical for myogenesis.[18] We found that the AF associated SNP at this locus is associated with reduced CAND2/TIP120b expression in striated muscle tissue. While the specific role of CAND2/TIP120b in AF is currently unclear, we observed atrial action potential prolongation by morpholino-mediated gene knockdown in the zebrafish. Additionally, our eQTL analyses indicate that the risk allele is associated with increased expression of CAND2. Extrapolating our findings in the zebrafish, increased CAND2 levels would be predicted to shorten the atrial action potential duration, as has been widely observed in AF.

GJA1, a strong candidate gene at our fourth AF locus, encodes the gap junction protein connexin 43 on chromosome 6q22.31 which is abundantly expressed in the heart.[19] We found that AF-associated SNPs influenced the transcription of *GJA1* in both left atrial tissue and the whole heart. Connexin 43 is the predominant cardiac gap junction protein and facilitates coordinated electrical activity between adjacent myocytes. Germline mutations in *GJA1* have been associated with syndromic diseases such as hypoplastic left heart syndrome, atrioventricular canal defects, or oculo-dento-digital dysplasia. Interestingly, a somatic, loss-of-function mutation in connexin 43 has been found to underlie AF in humans.[20] Further, mice with 60% reduced atrial *Gja1* expression showed an increased susceptibility to induced AF and atrial tachycardia.[21] Two independent swine models with an AF induced reduction of *GJA1* expression demonstrated that restoration of *GJA1* expression ameliorated AF burden.[22] 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.[25] Yet, the reported SNP rs1265564 only displays 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 as we did not find evidence for an association in the region among Europeans (Supplemental Figure 1). The specificity of the *CUX2* association in Japanese was in contrast to other four loci that were all associated with AF to varying degrees (Table 1 and Supplemental Table 7). The variability in the association between individuals of European and Japanese ancestry may be due to differences in allele frequency, sample size or another intrinsic difference between the populations.

Clinically, AF confers a five-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 two other loci conferred an increased risk of cardioembolic stroke. Given that two 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 two 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 three 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 both to increase the

generalizability and the statistical power of the current analyses. We acknowledge that the NEURL:PITX2 interaction that we observed was *in vitro* and further *in vivo* studies will be necessary. As with other GWAS, 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, co-immunoprecipitation, 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.

In summary, using a combination of genetic association, 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 further expand our understanding of the molecular pathways and clinical implications of this common and morbid arrhythmia.

Supplementary Material

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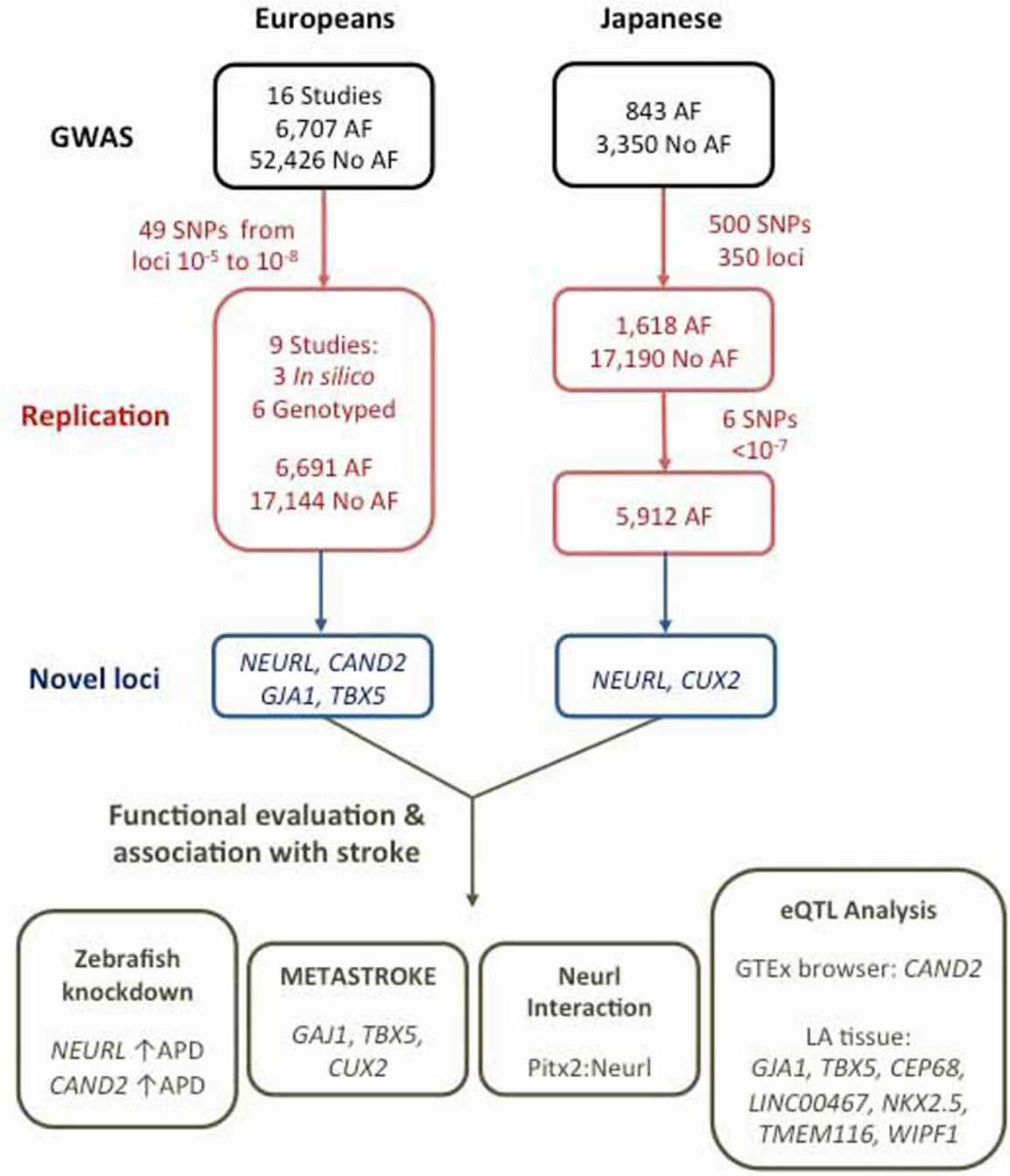

Figure 1.

Flow-chart illustrating the study design and major results. Novel chromosomal loci associated with AF were identified independently in cohorts of European and Japanese descent by means of 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*, an interaction between NEURL and PITX2, an association of *GJA1*, *TBX5*, and *CUX2* with stroke, and eQTL

associations with *CAND2*, *GJA1*, *TBX5*, *CEP68*, *LINC00467*, *NKX2.5*, *TMEM116*, and *WIPF1* in left atrial and other tissues. APD – action potential duration.

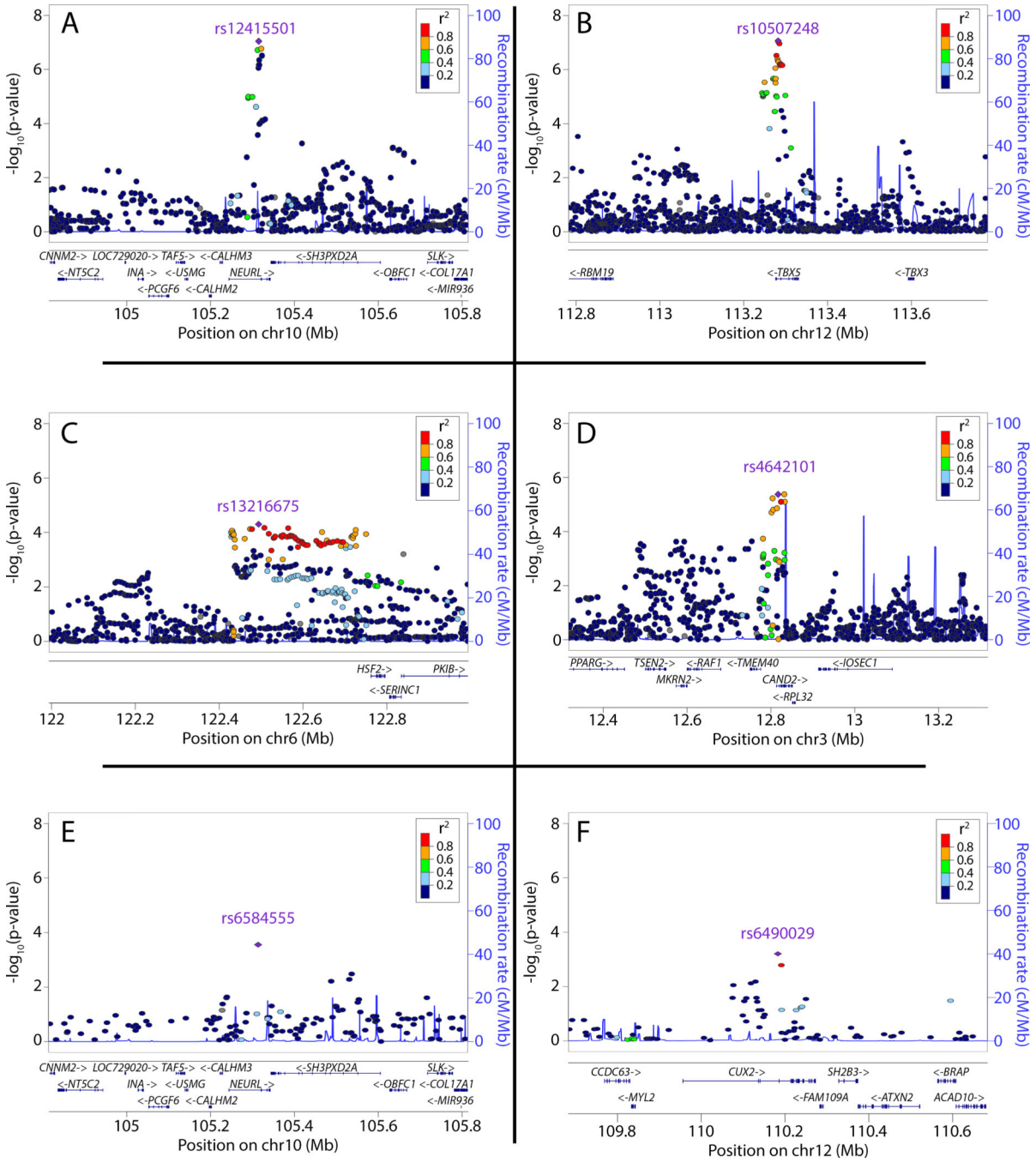

Figure 2.

Regional plots for novel atrial fibrillation susceptibility loci in Europeans and Japanese. Panels **A-D** (**A**: *NEURL* **B**: *TBX5* **C**: *GJA1* **D**: *CAND2*) show 4 novel loci detected in Europeans, panels **E** (*NEURL*) and **F** (*CUX2*) show 2 novel loci detected in Japanese. At each novel locus ($p \leq 5 \times 10^{-8}$), SNPs are plotted using the genomic position (NCBI Build 36) and discovery stage *P* values. In each panel, the sentinel SNP is labeled in purple. Each dot represents a 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, where red indicates

strong, and blue indicates weak linkage disequilibrium. Estimated recombination rates are shown by the blue line, and spikes indicate 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 panels **A-F** are based on the CEU HapMap release 22 (European) and JPT + CHB HapMap release 22 (Japanese), respectively. All regional association plots prepared using LocusZoom.

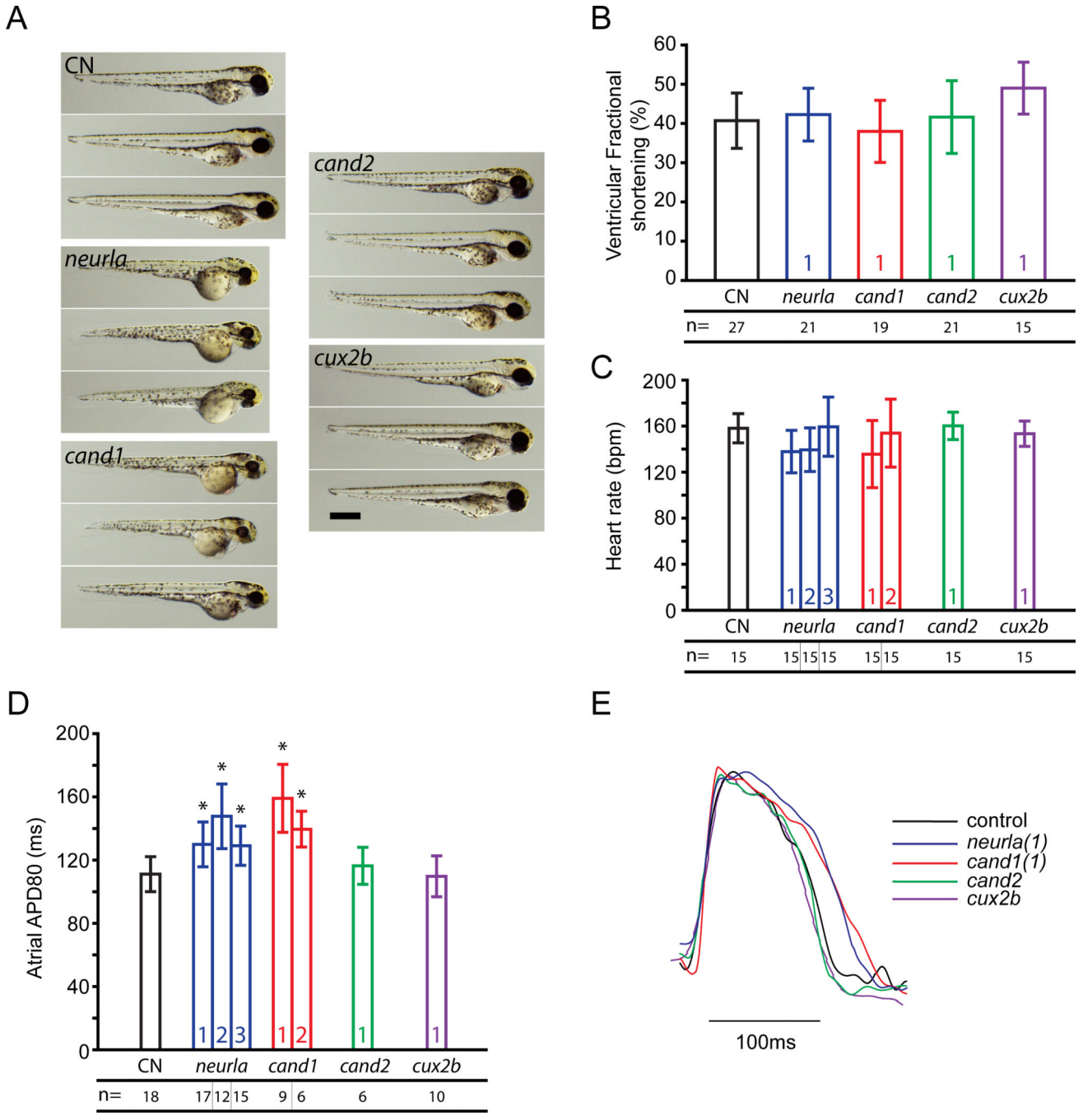

Figure 3.

Analysis of *neurla*, *cand1*, *cand2*, and *cux2b* knockdown in zebrafish. **A**: Brightfield micrographs of anesthetized 72hpf embryos injected with morpholinos. Scale bar = 500μm. **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. *Represents $p<0.05$ when compared to control. **E**: Representative traces of atrial action potentials from optical mapping. All numbers within bars indicate which morpholino was used for the presented data. Where no labels are shown, data represent pooled data obtained

from all effective morpholinos. CN – control. n=number of biological replicates for a given experiment.

Table 1

Meta-analyses of SNP-Associations with AF, by origin of study

SNP	Chr	AF Risk allele	Closest gene	Relative location	Original GWAS dataset[4]			Replication			Overall Meta-analysis		
					RAF	RR (95% CI)	p	RAF	RR (95% CI)	p	RAF	RR (95% CI)	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). SNP – single nucleotide polymorphism; AF – atrial fibrillation; Chr – chromosome; RAF – risk allele frequency; RR – relative risk; CI – confidence interval.

Table 2

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

Phenotype	Cases	Controls	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	2,365	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