**An atrial metastable state underlies the primary substrate in a genetic form of atrial fibrillation**

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## Summary Paragraph

**Atrial fibrillation (AF) is the most common clinical arrhythmia and is associated with heart failure, stroke and increased mortality[1]. The myocardial substrate for AF is poorly understood due to limited access to primary human tissue and the lack of mechanistically faithful *in vitro* or *in vivo* models[2, 3]. Using an *MYH6:mCherry* knock-in reporter line we developed a protocol to generate and purify human pluripotent stem cell-derived cardiomyocytes displaying physiological and molecular characteristics of atrial cells (hPSC-atrial cells). We modeled human *MYL4* mutants, one of the few definitive genetic causes of AF[4]. To explore non cell-autonomous components of AF substrate we also created a zebrafish *Myl4* KO model, which exhibited molecular, cellular and physiologic abnormalities that parallel those in humans bearing the cognate mutations. There was evidence of increased retinoic acid signaling in both hPSC and zebrafish mutant models, with abnormal expression and localization of cytoskeletal proteins, and loss of intracellular NAD and NADH. To characterize potential upstream mechanisms, we performed a chemical suppressor screen integrating multiple human cellular and zebrafish *in vivo* endpoints. This screen identified connexin 43 hemichannel (HC) blockade[5], as a robust suppressor of the abnormal phenotypes in both models of MYL4-related atrial cardiomyopathy. Immunofluorescence and co-immunoprecipitation studies revealed an interaction between MYL4 and Cx43 with altered localization of Cx43 HCs to the lateral membrane in *MYL4* loss of function mutants, as well as in atrial biopsies from unselected forms of human AF. The membrane fraction from *MYL4*-/- hPSC-atrial cells demonstrated increased phospho-Cx43 which was further accentuated by retinoic acid (RA) treatment and by risk alleles at the Pitx2 locus. Protein kinase C was induced by RA, and its inhibition also rescued the abnormal phenotypes in both atrial cardiomyopathy models. These data establish a mechanistic link between the transcriptional, metabolic and electrical pathways previously implicated in AF substrate and suggest novel avenues for the prevention or therapy of this important arrhythmia.**

**Introduction**

AF is the most common arrhythmia worldwide and is an independent risk factor for the development of heart failure, stroke and overall cardiovascular mortality[3]. AF management typically includes control of rapid rates, suppression of episodes of atrial arrhythmia and anticoagulation to reduce the incidence strokes and other thromboembolic events, which are a major complication in a subset of AF cases[6]. Many drugs have been developed to suppress atrial fibrillation, but few exhibit significant advantage over placebo controls and the majority have narrow therapeutic windows or significant side effects[7]. Increased excitability of pulmonary venous smooth muscle cells is know to be a driver in a subset of patients with isolated AF and this led to the development of percutaneous methods to electrically isolate the pulmonary veins from the left atrium. Nevertheless, it has proven challenging to demonstrate definitive evidence of durable effects of ablative technologies on disease outcomes[8]. The thromboembolic complications of AF have been attributed to rheological stasis in the setting of loss of atrial contractility, but the risk of stroke is heterogenous and there is also an increased baseline risk for hemorrhagic stroke which suggests more complex mechanisms.

The limited success of pharmacological approaches to AF is at least in part a consequence of the inaccessibility of AF mechanisms, as human left atrial tissue is rarely available prior to the onset of the arrhythmia or data are confounded by chronic left atrial abnormalities[9]. There are also no robust mechanistically faithful in vitro or in vivo AF models[10]. The most common animal models exploit chronic rapid atrial pacing, typically in goats or sheep, to mimic and then induce AF which persists only when pacing is continued[2]. These studies have demonstrated that rapid atrial rates predispose to subsequent AF, they have reproduced the molecular findings in human tissue collected after the onset of AF and they have rigorously reinforced the clinical concept that "AF begets AF". However, these 'disease' models do not represent any known primary AF mechanisms, do not appear to predict human pharmacological responses and typically revert to sinus rhythm on cessation of rapid atrial pacing[11].

Genetic approaches to the mechanisms of AF have been slowed by reduced penetrance, but there is a substantial recurrence risk within families, suggestive of significant large effect size alleles[12]. Genome wide association studies have identified multiple loci, although the mechanisms remain unknown and effect sizes mitigate against efficient drug discovery[11]. Rare Mendelian forms of AF

exist, but are often confounded by ventricular dysfunction with secondary effects on the atrial phenotype[13]. Many AF loci overlap with known loci for non-ischemic cardiomyopathy and AF is a prominent feature of many of these inherited forms of heart failure, sometimes prior to overt ventricular disease[14]. Heritable lone AF (without any echocardiographic evidence of structural abnormality or ventricular dysfunction) has been described in multiple unusual kindreds including a variant of the long QT syndrome with specific gain of function mutations in KCNQ1 and a congenital form of AF associated with childhood sudden death and mutations in the nuclear membrane pore protein NUP155[15]. Recently, genetic studies in Icelandic populations led to the discovery of an inherited form of more typical AF caused by loss of function mutations in the MYL4 gene[4]. This form of AF is characterized by autosomal dominant or co-dominant inheritance, early onset of AF, stroke and the absence of ventricular dysfunction in atrial cardiomyopathy (AC) patients harboring MYL4 frameshift mutations[16].

Given the overlap in phenotypes between MYL4-associated AF and a substantial subpopulation within typical AF cohorts, we explored the mechanism of this form of the arrhythmia. We established and optimized a protocol for deriving human atrial lineages from human pluripotent stem cell (h-PSC) and characterized it at single cell level. A truncation mutant in MYL4, recreating the human mutant peptide, was generated in hPSC derived atrial cells to enable the assessment of cell-autonomous components of the pathogenesis of this form of AF. To assess non cell-autonomous components of MYL4 associated AF substrate we created the cognate germline mutations in a zebrafish model. Together these human cellular and animal models were chosen to undertake chemical or genetic modifier or suppressor screens which were performed in parallel to increase the specificity of the consequent findings. Initial characterization of these models revealed the emergence of a discrete population of retinoic acid synthesizing cells, as well as distinctive cytoskeletal and metabolic defects. In addition, we were able to establish a direct genetic interaction between MYL4 effects and the most common risk allele for AF at the PITX2 locus. An exploratory chemical suppressor screen identified blockade of the Cx43 hemichannel as a robust mechanism for rescue of the core disease phenotypes in both cellular and zebrafish models and this was subsequently validated using multiple independent approaches. Finally, we were also able to identify perturbations of PKC signaling which when reversed could also rescue cellular and cardiac abnormalities. These data define subtle perturbations of cardiomyocyte differentiation and coupling, via classical epithelial mesenchymal transition pathways as a mechanistic link between

the transcriptional, metabolic and electrical pathways previously implicated in AFib. This work also identifies novel targets for the suppression of the major discrete components of this complex multifaceted disease.

**Single cell characterization of human atrial hPSCs**

To assess the differentiation efficiency and facilitate prospective purification of atrial cardiomyocytes, an *MYH6:mCherry SHOX2:GFP* knock-in reporter line was created in the parental H9 hESC background using CRISPR/Cas9-based gene-targeting techniques. The mCherry reporter signal co-localized with cardiac structural proteins, including cTnT and cardiac Actin (Supplementary Figure 1A, B), and enabled the development of protocols to optimize the generation of hPSC-atrial cells. Based on previous studies[17-19], we used a stepwise differentiation protocol, with initial mesoderm induction followed by direction of the identity of cardiac progenitors toward a more caudal phenotype (Figure 1A). To assess the phenotypic fidelity of the resultant hPSC-atrial cells, we sorted mCherry+ cells and measured action potentials in current clamp mode, revealing features consistent with mature atrial electrophysiology (Figure 1B). At day 60, hPSC-derived cells stained positive by immuno-fluorescence for MLC2-a, cTnT, cardiac actin, MYL4 and negative for MLC2v (Figure 1C). No GFP+ cells were detected in the FACS analysis, excluding a nodal identity for these cells (Supplementary Figure 1C).

We performed single-cell RNAseq analysis on differentiated hPSC-atrial cells. To minimize batch to batch variation, we sorted the cells according to mCherry expression and mixed positive and negative fractions in fixed ratios prior to barcoding to minimize inter-sample variation (Figure 1D and Supplementary Figure 1D). The *MYH6*+ population also expressed atrial specific markers including *NPPA*, *MYL4*, *MYL7* (MLC2a) while the entire atrial differentiation population (including the MYH6+ majority) lacked ventricular, conduction system, or epicardial markers such as *MYL2*, *NPPB*, *CNTN1*, *IRX4, HEY2, ISL1, TBX18* and *MYH11* (Figure 1E-G and Supplementary Figure 1E-F). The top 20 transcripts in the MYH6+ population included discrete markers proposed for human atrial cell annotation such as *NR2F2*[20, 21] (Supplementary Figure 1E), among which MYH6 exhibited a more consistent overlap with other known atrial-specific genes (such as MYL4 and NPPA) and therefore was chosen for lineage marking (Supplementary Figure 1F ).

**MYL4 mutant cell lines reveal increased retinoic acid synthesis and actin disorganization**

We generated disease cognate mutations in MYL4 using CRISPR-Cas9 technology in hESCs (Figure 2A and Supplementary Figure 2A,B) to study this form of AF substrate. scRNA-seq on *MYL4*+/- and *MYL4*-/- derived hES-atrial cells at day 60 was compared to that from *MYL4*+/+ controls (Figure 2C). Cells derived from both wild-type and mutant lines included populations expressing atrial lineage markers (Supplementary Figure 3A). However, the transcriptional data also identified a discrete population of cells in mutant lines significantly enriched in the retinoic acid synthesis and downstream retinoic acid response pathway members, including *ALDH1A2, RDH10, EDN3* and *KLK5*. (Figure 2D and E, Supplementary Figure 3B and C). This transcriptional phenotype was further accentuated in the context of the known common *PITX2* AF risk allele rs2200733[22, 23] (Figure 2E and Supplementary Figure 3C) which has been postulated as a general modifier of AF substrates[24]. FACS analysis revealed increased percentages of cells expressing ALDH1A2 and RDH10 in the *MYL4*-/- compared to *MYL4*+/+ with additional increases observed in *MYL4*-/- bearing the rs2200733 allele. The changes observed at cellular level in the hPSC-atrial model were also observed in Myl4 knock-out zebrafish lines generated on the previously published nppb:Luc background (Figure 2F and Supplementary Figure 3E). Specifically, immunofluorescence revealed increases in RDH10 expression at 5dpf and RNAseq and ATACseq of 3dpf hearts further confirmed *in vitro* activation of retinoic acid synthesis in the zebrafish model (Figure 2G and h and Supplementary Figure 3F).

Next, we focused on the differentially expressed genes between wild-type and mutant lines within the MYH6+ hPSC-atrial cells. The mutant cells expressed key atrial lineage markers (Figure 2I and Supplementary Figure 4). However, a number of genes associated with familial forms of cardiomyopathies that are linked with actin cystoskeleton remodeling and organization were differentially expressed (Figure 2J and Supplementary Figure 4). We confirmed MYL4-actin interaction by showing that recombinant MYL4 protein could activate pyrene-actin polymerization as efficiently as two known potent activators, Arp2/3 and VCA (Figure 2K). Next, we tested MYL4 and actin cytoskeletal distribution in heart biopsies from individuals in normal sinus rhythm, demonstrating significant colocalization. This co-localization was significantly disrupted, with evidence of increased membrane localization of MYL4, in the left atrial biopsies from those undergoing cardiac surgery who developed AF postoperatively and from those with known AF, but not in control subjects who did not develop AF (Figure 2L-N and Supplementary Figure 5).

**Chemical screening to identify proximate pathway modifiers**

There are numerous potential pathways by which MYL4 loss of function might predispose to AF through alterations in retinoic acid signaling or cytoskeleletal remodeling, so to prioritize subsequent investigation we performed a chemical suppressor screen, with a library of known bioactive molecules, using a series of physiologic, cellular and molecular endpoints in both hES-atrial mutant cell lines and zebrafish *Myl4* mutant models.

Homozygous Myl4 mutant fish exhibited significantly lower heart rates and lower *Nppb*::luciferase levels (as well as consistent ATAC-seq; Figure 3A and Supplementary Figure 6A), with conserved ventricular function consistent with clinical data from *Myl4* mutant kindreds. Physiologic screening endpoints in fish included heart rate, contractility, blood flow and nppb::luciferase levels (Supplementary Figure 3A and 6B), as these are representative of the human disease and were observed to reproducibly discriminate between mutant and WT animals. In hES-atrial mutant cells the endpoint used was the percentage of RDH10+ cells (Figure 3B). While there were multiple partial hits in each screen, parallel unbiased screening in duplicate using the same chemical library in human mutant hES-atrial cells and zebrafish identified only one shared high fidelity hit: carbenoxelone (Figures 2C-E, Supplementary Table 1 and Supplementary Figure 6B-D).

While the molecular targets of carbenoxolone are not fully understood, there are well described effects blocking connexin hemichannels(HCs) at high affinity[25, 26]. Given these findings, we explored a potential primary role for HC physiology in AC caused by MYL4. Both IF and EM revealed evidence of abnormal trafficking of Cx43 in human and zebrafish MYL4 loss of function mutants (Figure 4A and B, Supplementary Figure 7A). Furthermore, biochemical pull-down confirmed that MYL4 is associated with Cx43 and actin in both wild-type and mutant D60 hES-atrial cells (Figure 4C, full blots in Supplementary Figure 7B). Both actin filaments and microtubules are known to be involved in Cx43 trafficking in cardiomyocytes[27]. Cx43 localization abnormalities were identified in the right atrial biopsies from patients with post-op AF and from those with permanent AF, but not in control subjects with sinus rhythm. Immunostaining of the biopsies for Cx43, ZO-1 and Actin, revealed relocalization of Cx43 to the lateral membrane and its reduced colocalization with actin filaments. (Figure 4D and E and Supplementary Figure 7C).

**Cx43 hemichannel blockade reverses metabolic and electrical changes in mutant lines**

Cx43 HCs are present in non-junctional membrane and play a role in transmembrane signaling that is dependent on their phosphorylation status[28, 29]. Conducting HCs allow ions as well as metabolites to transit, though gating mechanisms must exist to prevent the dissipation of transmembrane electrical gradients. We compared the cellular metabolite profiles of D60 hES-atrial cells derived from *MYL4+/+* or *MYL4-/-* lines and observed significant differences in the levels of multiple metabolites, including NAD and NADH (Figure 4F and Supplementary Figure 8A). There was no difference in the level of nicotinamide riboside, the precursor of NAD in these cells, suggesting that the differences observed are not a consequence of precursor bioavailability. Quantitative NAD measurement revealed higher levels of NAD in rescued mutant atrial cells than in untreated mutant controls (Figure 4G). The specific Cx43 HC blocker, Gap19, which does not exhibit gap junction blocking effects, restored intracellular NAD concentrations in mutant cells (Figure 4H).

Tight control of Cx43 HC permeability is necessary for normal electrophysiologic function[30]. Current-clamp measurement of action potentials from hES-atrial cells demonstrated a significant change in voltage amplitude and change over time, both reversible with carbenoxolone treatment (Figure 4I). No changes were observed in maximum diastolic potential (Supplementary Figure 8B). We tested whether HC blockade was able to rescue whole heart electrophysiological changes in Myl4 mutant fish. First, 3dpf zebrafish hearts were isolated and labeled with voltage sensitive dye (Fluovolt) for optical mapping (Figure 4J). *MYL4-/-* atria showed significantly prolonged action potential duration (APD) compared to WTs. By contrast, there was no difference in conduction velocity (Supplementary Figure 9A), which is in part determined by gap junction dependent intercellular coupling. The effects of MYL4 loss of function on APD were reversible with two specific Cx43 HC blocking peptides, Gap19 and Gap26 which also did not affect conduction velocities (Supplementary Figure 9A).

Calcium handling abnormalities are among the earliest features of AF and have been associated with the myocardial remodeling that sustains the arrhythmia[31]. Isolated hearts were stained with the calcium dye, Fura2, and atrial [$Ca^{2+}$] transients were measured using high-speed ratiometric calcium imaging (Figure 4K). Calcium transient amplitude, but not transient duration was increased in *Myl4-/-* zebrafish hearts (Supplementary Figure 9B) and rescued by Cx43 HC blockade. The Gap26 peptide, which binds the extracellular domain of the Cx43 HC, (in contrast

to Gap19 which binds intracellularly), only partially rescued this component of the mutant phenotype. These data suggest the increased calcium transient amplitude associated with perpetuation of AF is also a core component of the primary substrate in at least some inherited forms of the arrhythmia[32].

**PKC mediates of Cx43 hemichannel phosphorylation in MYL4 mutants**

Cx43 HC permeability is determined by phosphorylation state[29], and bulk RNA-seq revealed increased expression of multiple protein kinase genes in D60 hES-atrial cells derived from mutants when compared with cells derived from wild-type controls (Figure 5A). The Cx43 in the membrane fraction of MYL4 mutant cells was hyperphosphorylated, compared with the cytoplasmic fraction (Figure 5B). Culturing cells on surfaces with higher surface tension to model the effects of chronic stretch or the combination of MYL4 loss of function with the rs2200733 C-T PITX2 AF risk allele, result in further increases in phosphorylated Cx43 in the membrane fraction (Figure 5C and D). Multiple phosphorylation sites in the C-terminal cytoplasmic domain of Cx43 regulate hemi-channel permeability, regulated by multiple kinases (Figure 5E)[33, 34,35]. Previously, higher CAMKII activity has been demonstrated in atrial samples from chronic AF, but not from paroxysmal AF animal models[36]. CAMKII inhibition in our models exaggerated the prolongation of the abnormal APD in mutant cells, while both PKC and PKA inhibition partially normalized APD (Figure 5F). While neither PKA nor CAMKII inhibition were able to significantly reduce calcium amplitude in mutants, PKC inhibition resulted in the reversal of calcium transient amplitude changes without affecting transient duration (Figure 5G and Supplementary Figure 9C). PKC inhibition also decreased Cx43 hyperphosphorylation and rescued intracellular NAD levels in mutant hES-atrial cells (Figure 5H and I). In contrast, treating mutant hES-atrial cells with retinoic acid accentuates Cx43 phosphorylation and PKC activity (Figure 5H and J). Taken together, these results suggest a role for PKC activity, at least in part through Cx43 modulation, in this form of AF.

**Discussion**

AF is observed in numerous disease syndromes ranging from primary myocardial disease to sepsis, but its characterization has been complicated by limited access to the primary atrial substrate before AF itself results in secondary findings[37]. To identify proximate mechanisms for AF, we modeled both the cell-autonomous and non cell-autonomous components of a genetic form of AF substrate (prior to AF) in systems suited to subsequent unbiased screening. Focusing on loss of function mutations in the MYL4 gene, one of the few identified heritable causes of AF[4], we chose to characterize the features of this specific entity using mutant hES-derived atrial cells and mutant zebrafish lines in parallel. In addition, to anchor our findings definitively in human AF biology, we exploited a unique set of atrial biopsies obtained from patients during surgery prior to the emergence of a discrete form of AF occuring in the context of a substantial proportion of cardiac surgical procedures.

We first developed and optimized protocols to enable the characterization of atrial lineages derived from human pluripotent stem cells at the single cell level in both health and disease. By introducing mutations in MYL4 we were able to study single cell structural, electrophysiologic and transcriptional features of AF substrate. Using a cognate approach in the zebrafish, we could also explore tissue and organ level physiology during similar stages of differentiation. Together these approaches offer efficient and powerful insights into the biology of this genetic form of human atrial disorder.

The initial insights from these models is the emergence of a specific population of retinoic acid (RA) synthesizing cells in the setting of mutant MYL4. While scRNAseq can result in spurious cell populations, we were able to mitigate this risk using a controlled pooling strategy, but also used germline zebrafish MYL4 mutants to confirm these findings *in vitro*. Interestingly, RA is known not only to be a necessary signal for development and patterning[18], but in multiple systems regulates regional membrane biology and cell-cell interaction through differential phosphorylation of a range of protein kinases [38]. In the atrium these changes would be predicted to cause significant changes in cell physiology dependent on cell polarity[39]. Multiple molecular pathways have been implicated in the maintenance of cellular polarity, including Wnt-beta catenin, TGF-beta and calcium handling and the cytoskeleton[40-42],but these pathways have diverse effects so we continued

to follow unbiased approaches to understanding the mechanisms of MYL4 mutation in AF and related myocardial diseases.

Single cell RNAseq of mutant hPSC-atrial cells also identified significant perturbation in cytoskeletal gene expression and we subsequently demonstrated that MYL4 interacts with cytoskeletal actin *in vitro* and *in vivo*. In mutant cells from hPSC-atrial lines and in human atrial biopsies from those who subsequently developed AF, we observed abnormal relocalization of MYL4 to the lateral membrane with relative loss of colocalization of the signal with actin. These findings, and mislocalization of actin and MYL4 compared with the junctional marker ZO-1, further supported abnormal polarization of the cardiomyocyte cell membrane in both the cellular model and in human disease samples.

In a systematic effort to identify proximate mechanisms in our two AF substrate models we performed unbiased screens of known bioactive small molecule libraries for compounds capable of suppressing the abnormal phenotypes. These parallel screens identified carbenoxelone as the only compound in this limited set capable of suppressing all of the phenotypes in both models. Given carbenoxelone's prior annotation as a blocker of connexin hemichannels[25, 26] we then tested this mechanism as the mode of suppression. We first characterized extensively physiologic changes in both human cellular and zebrafish MYL4 mutants, demonstrating abnormalities in cellular electrophysiology, calcium handling and cellular energy substrate leak. We found that not only did HC blockade explain the rescue we had observed, but also demonstrated that other abnormalities, including NAD/NADH leak, were also consistent with pathological increases in Cx43 HC density and conductance in the lateral membrane[28]. Notably these features were not associated with any electrical coupling abnormalities, further reinforcing the specificity of the hemichannel phenotype. We were able to confirm the presence of increased lateral membrane hemichannel expression in human tissue samples from those who were in sinus rhythm, but subsequently developed AF, implicating a partial change of cellular organization phenotype as an upstream substrate for a subset of AF. We also established a significant genetic interaction between the MYL4 mutant allele and the largest effect size common risk allele for AF at the PITX2 locus across multiple cellular outputs.

HC permeability is known to be regulated by both location and phosphorylation status in other tissues. We found that among protein kinases with a potential target site on Cx43, PKC inhibition

resulted in significant reduction in phosphorylation status as well inhibition of membrane permeability to NAD/NADH and perturbations in calcium and voltage homeostasis. Retinoic acid is also able to directly increase PKC activity, a process which was reversible with RA antagonist. Thus, not only does the Cx43 HC appear to be an attractive candidate target for suppression of this form of AF substrate, but existing PKC inhibitors, already known modulators of cell coupling, may also be a viable adjunct.[34]

Together these data strongly support a graded and subtle form of cell polarity perturbation as the underlying mechanism for MYL4 related AF. Indeed several observations are consistent with atrial myocardium occupaying a metastable state at baseline which can be disrupted by factors which which increase cardiomyocyte differentiation (exercise, stretch, retinoic acid) or decrease cardiomyocyte differentiation (MYL4 mutation, thyroid hormone, PITX2 locus, obesity). This deviation from an intrinsically metastable state may reflect the balance between adaptability (e.g. for mechanotransduction) and disease, and is consistent with the large number of inputs that result in this arrhythmia affecting up to 10% of all humans. Interestingly, using the OPEN algorithm we have previously published to identify the most likely genes at GWAS loci, those for the top AF GWAS loci all participate in a large network which is dominated by genes involved in cell polarity (data not shown; http://cvri.ucsf.edu/~deo/disease_mapping.html).

In summary, we identified perturbation of atrial myocardial cell polarity as a shared upstream mechanism integrating electrical, metabolic and cellular structural abnormalities in MYL4 related AF substrate. Using an unbiased high-throughput screening platform that combined several physiologic and molecular endpoints, we identified the Cx43 HC as a key target that may offer an avenue for antiarrhythmic therapy that complements traditional ion channel blockade. These insights imply that many of the downstream features may be a consequence not just of the sustained burden of AF, but rather of the primary mechanism itself and raise the possibility that endothelial cells, adipocytes, fibroblasts and other cell types may each contribute through related perturbations of their basal differentiation. Additional work in a range of experimental and clinical settings will be required to test this hypothesis, but the possibility for precision interventions for some of the forms of AF is evident.

**Methods**

**Cell culture**
hESC line H9 and the derivative (MYH6:mCherry, MYL4-/-, MYL4+/-, MYL4-/-rs2200733 SNP) line were grown on Matrigel-coated plates and maintained in mTeSR medium (Stem Cell Technologies).

**Atrial lineage induction**
Differentiation was initiated 72 hours after plating when the culture was approximately 80% confluent. Cells were first differentiated toward a mesoderm phenotype subsequent to treatment with 1.5 µM CHIR99021 (CHIR, Stem-RD), 20 ng/mL BMP4 and 20 ng/mL Activin A in RPMI (Cellgro) supplemented with B27 minus insulin, 2 mM GlutaMAX, 1x NEAA and 1x Normocin (InvivoGen) for 3 days (RB27-INS). Cells were caudalized toward an atrial phenotype by treatment with 1 µM retinoic acid (Sigma Aldrich) from day 3-6 in RB27-INS. 5 µM XAV939 was added from day 4-6. From day 6 onward, differentiation of cells was carried out in RPMI supplemented with B27, 2 mM GlutaMAX, 1x NEAA and 1x Normocin (RB27+INS).

**Single cell RNA sequencing**
*Encapsulation and library preparation.* Cells were encapsulated at the Harvard Medical School Single Cell Core (https://singlecellcore.hms.harvard.edu/) using the inDrops v3 library format[43]. Reverse transcription and library preparation were performed at the same facility. For inDrops-seq, the cells were encapsulated in droplets and the libraries were made following a previously described protocol[43], with the following modifications in the primer sequences.

RT primers on hydrogel beads:
  5'-
CGATTGATCAACGTAATACGACTCACTATAGGGTGTCGGGTGCAG[bc1,8nt]GTCTCG
TGGGCTCGGAGATGTGTATAAGAGACAG[bc2,8nt]NNNNNNTTTTTTTTTTTTTTTTTTTT
V-3'
R1-N6 primer sequence (step 151 in the library prep protocol in[43]):
  5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNN-3'
PCR primer sequences (steps 157 and 160 in the library prep protocol in[43]):
  5'-AATGATACGGCGACCACCGAGATCTACACXXXXXXXXXTCGTCGGCAGCGTC-3',
where XXXXXX is an index sequence for multiplexing libraries.
  5'-CAAGCAGAAGACGGCATACGAGATGGGTGTCGGGTGCAG-3'

With these modifications in the primer sequences, custom sequencing primers are no longer required.

*Sequencing.* Prior to sequencing, the fragment size of each library was analyzed on a Bioanalyzer high sensitivity chip. Libraries were diluted to 1.5 nM and then quantified by qPCR using primers against the p5-p7 sequence. inDrops libraries were sequenced on an Illumina Nextseq 500 with following parameters: (1) 61 bp read 1; (2) 8 bp index 1 (i7); (3) 8 bp index 2 (i5); (4) 14 bp read 2. Binary base call (BCL) files were converted into FASTQ format with bcl2fastq, using the following flags that are required for inDrops v3: (1) `--use-bases-mask y*,y*,y*,y*`; (2) `--mask-short-adapter-reads 0`; (3) `--minimum-trimmed-read-length 0`.

***Count matrix generation.*** The software versions used to generate counts from the FASTQ files were managed with bcbio-nextgen 1.0.6a0-d2b5b522 (https://github.com/bcbio/bcbio-nextgen) using bioconda (https://bioconda.github.io/). First, cellular barcodes and UMIs were identified for all reads. Second, reads with one or more mismatches to a known barcode were discarded. Third, remaining reads were quasi-aligned to the *Homo sapiens* GRCh38 (Ensembl 90) reference transcriptome using RapMap 0.5.0 (https://github.com/COMBINE-lab/RapMap). Reads per cell were counted using the umis 0.6.0 package for estimating UMI counts in transcript tag counting data[44] (https://github.com/vals/umis), discarding duplicated UMIs, weighting multi-mapped reads by the number of transcripts they aligned to, and collapsing counts to genes by adding all counts for each transcript of a gene. Finally, cellular barcodes with fewer than 1,000 reads assigned were discarded from the analysis (see "minimum_barcode depth" in bcbio documentation for details).

***Quality control analysis and filtering.*** Gene-level counts were imported to R using the bcbioSingleCell 0.1.8 package (https://github.com/hbc/bcbioSingleCell). This package extends the Bioconductor SingleCellExperiment container class, which is optimized for single-cell RNA-seq (https://bioconductor.org/packages/SingleCellExperiment/). Quality control analysis was performed using this package, and the `filterCells()` function was used to filter out low quality cells by keeping cellular barcodes with the following metrics: (1) >= 1000 UMIs per cell; (2) >= 500 genes per cell; (3) >= 0.8 novelty score, calculated as log10(genes detected) / log10(UMI counts per cell). Additionally, genes with very low expression across the data set were filtered by applying a cutoff of >= 3 cells per gene. After initial quality control, we maintained a total of 6,074 cells and 21,576 genes; WT samples alone accounted for 1,784 cells with 21,576 genes.

***Clustering and marker analysis.*** Clustering of our filtered, high quality cell data set was then performed using the Seurat 2.1 package[45]. This step includes (1) log2 normalization and transformation/scaling of the UMI counts per cell to account for technical differences in sequencing depth; (2) identification of highly variable genes using the FindVariableGenes() function with default parameters for dimensionality reduction; (3) regression of sources of unwanted variation, specifically by adjusting for the variable number of RNA molecules captured per cell (nUMI) and phase/cell cycle (genes used that comprise the S.Score and G2M.Score are provided in the Github repository); (4) for WT only analysis, identification of primary sources of heterogeneity using principal component analysis (PCA) and clustering of cells based on the top 15 significant PCs (meta-genes); and (5) for combined analysis of the WT and MYL4 KO samples, canonical correlation analysis (CCA) adjustment and clustering of the cells using the top 25 aligned dimensions (CCs). Clusters were identified using the shared nearest neighbor (SNN) modularity optimization with a clustering resolution set to 0.6. This resulted in 8 WT clusters and 12 clusters for the combined set of WT and KO samples.

Specific gene markers for each cluster were identified with the FindAllMarkers() function using the Wilcoxon rank sum test. Non-linear dimensionality reduction using t-distributed stochastic neighbor embedding (t-SNE) and uniform manifold approximation and projection (UMAP)[46] was performed to visualize and explore the data. Cluster quality was assessed for possible cluster artifacts (variance correlated with UMI counts, mitochondrial ratio, batch effects, and any other principle component biases). Additional differential expression at the gene level between clusters was performed with DESeq2 1.20 (https://bioconductor.org/packages/DESeq2/).

***Data files and analysis code.*** The original FASTQ files, UMI-disambiguated counts in MatrixMarket Exchange format (MTX files) [see https://math.nist.gov/MatrixMarket/info.html for details], and inDrops v3 sample barcodes used are available on the Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA) [GEO: <processing>; SRA: <processing>]. The code used to perform clustering and marker analysis is available on GitHub (https://github.com/hbc/hbc_macrae_cardiomyocytes_singlecell_hsapiens).

## Immunofluorescence (IF)

For *in vitro* cultured human cells: Culture medium was aspirated and the cells were washed 3x with PBS. Next, PFA4% was added to the well and incubated for 20 minutes at 4 degrees C. After washing the cells 3x with PBS, they were permeabilized and blocked in 0.3% Triton-PBS with 2% horse serum (perm-block buffer) for 1 hour at room temperature before incubation with primary antibody in perm-block buffer overnight at 4 degrees. On the following day, they were washed 3x with PBS and secondary antibody was added to perm-block buffer and incubated with the cells for 1 hour at room temperature followed by DAPI staining and 3x wash with PBS.

For zebrafish: Zebrafish embryos at 3dpf were transferred to Tyrode's solution (TS) containing Na+ (136), K+(5.4), Mg2+(1.0), PO43-(0.3), Ca2+(1.8), Glc (5.0), HEPES (10.0) (all in mM), and 1%BSA and hearts were isolated on the stage of an inverted microscope. Isolated hearts were transferred to fresh TS and then transferred to Prefer fixative (Anatech Ltd). The remaining protocol was as outlined for the *in vitro* cultured cells as described above. A list of primary antibodies used in this study can be found in supplementary table 2.

## FACS and intracellular staining

For sorting: cells were treated with Accutase (Innovative Cell Technologies) for 15 min at 37°C and neutralized using sorting medium (DMEM+5% FBS; both Thermo Fischer). Pellet was washed 2x with PBS and cells were reconstituted in PBS+2.5% BSA for sorting.

For intracellular staining: subsequent to pellet wash, cells were fixed using the FOXP3 Fixation/Permeabilization set (Thermo Fischer). After spinning down the fixed cells, they were washed, blocked and permeabilized using FOXP3 permeabilization buffer (Thermo Fischer) according to the manufacturer's protocol. Fixed and permeabilized cells were incubated with primary (overnight at 4 degrees) and secondary (45 mins, at room temperature) antibodies and analyzed using a flow cytometer.

## Generation of the MYH6:mCherry GFP:SHOX2, MYL4 -/- ± rs2200733 SNP hESC lines

The MYH6:mCherry GFP:Shox2 line was generated as previously described (manuscript in revision). Briefly, for targeting the MYH6 and SHOX2 loci, sgRNA sequences were designed using the website http://crispr.mit.edu/. sgRNA was cloned into the PX459 vector (Addgene, plasmid # 42230). For constructing the MYH6:mCherry donor plasmid, the mCherry (from Clontech), codon optimized P2A, and left and right homology arms (from H9 genomic DNA) were PCR amplified and assembled using the In-Fusion kit (Clontech) and then cloned into the zero-blunt plasmid (Thermo Fisher). The same strategy was used to generate SHOX:GFP donor plasmid using NLS-eGFP (from Tol2kit plasmid) to PCR amplify the corresponding fluorescence sequence.

To construct the rs2200733 donor plasmid, 180bp gDNA blocks with 90nt flanking homology arms around the SNP were synthesized (IDT). The dsDNA fragment was cloned into the zero-blunt plasmid.

For MYL4 knock-out lines, 2gRNAs spaced at <50nt were designed to target exon 4 of human MYL4 gene. gRNAs were cloned into PX459.

After confirming the insertion of homology sequence donor or gRNA sequence into the corresponding vector, cells were prepared for electroporation. H9 hESCs cultures were dissociated into single cells with Accutase. After 1x PBS wash, cells were re-suspended in 100 electroporation buffer containing 2 µg CRISPR targeting plasmid and 4 µg donor plasmid (for homologous recombination only) and electroporated using human stem cell nucleofector kit2 (Lonza, #VPH-5022) according to the manufacturer guidelines. Cells were transferred onto Matrigel coated plates with ROCK inhibitor. Two days after electroporation, transfected cells were selected by treatment with puromycin (0.5 µg/mL) for two days. Colonies surviving the treatment were picked and expanded for PCR genotyping and sequencing for the reporter lines.

The sequences for all primers used for generating these genetically modified lines are listed in Supplementary Table 3.

**Generation of the MYL4 loss of function zebrafish lines**
gRNA sequences were designed using the website http://chopchop.cbu.uib.no/. They targeted cmlc1 and MYL4. For each gene, 2 gRNAs spaced at <50nt were designed. The sequences are available in Supplementary Table 3. Briefly, crRNA and tracrRNA were ordered (IDT) and annealed to form duplexes in thermal cycler according to manufacturer's instructions. crRNA:tracrRNA duplex was incubated with Alt-R Cas9 Nuclease to form ribonucleoprotein (RNP) complex. 1.5nL of the mix was injected into the zebrafish embryos at the 1-cell stage. F0 fish were outcrossed onto TuAB fish line to identify homozygotes or heterozygotes.

Human MYL4 has two orthologs in Zebrafish, *Cmlc1* and *Myl4*. A recent paper studied the effect of *cmlc1* mutation on atrial pathology. Others have shown that *Cmlc1* is expressed solely in the ventricle in developing zebrafish embryos. Consistent with the previous findings, loss of function mutations in *cmlc1* resulted in zebrafish with atrophic, hypo-contractile ventricles (data not shown). We proceeded to test Myl4 as the most robust fish ortholog of human MYL4.

F0 fish were outcrossed to find F1 heterozygous individuals (*Myl4*+/-) and they were in-crossed subsequently to generate F2 *Myl4*-/- fish. F2 homozygous *Myl4*-/- fish were viable and reproduced without any abnormal gross morphology.

**Zebrafish heart ATACseq analysis**
25 Zebrafish hearts (~25k cells) were dissected as described above. To profile open chromatin status, we used ATACseq protocol as previously described. Briefly, hearts were centrifuged at 500 xg for 5 minutes at 4 degrees (x2) and 50 uL of cold lysis buffer was added to the pellet after aspirating supernatant PBS. The nuclear fraction was spun down at 500 g for 10 minutes and was resuspended in transposition reaction mix (containing 12.5 uL TD, 1.25 uL TDE1 and 11.75uL Nuclease Free water) followed-by incubation at 37 degrees for 30 minutes. The DNA mixture was immediately purified on a Qiagen MinElute PCR purification column (Qiagen) and proceeded to

PCR amplification step. After an initial extension step, additional number of PCR cycles was determined as number of cycles that corresponds to ¼ of maximum fluorescent intensity. After completion of PCR reaction, DNA fragments were purified on Qiagen MinElute PCR purification column and submitted for sequencing.

**Whole cell patch and current clamp**
Current-clamp experiments in atrial myocytes to measure transmembrane action potentials were carried out using standard protocols[47]. Micropipettes had a tip resistance of 2 to 5 megOhms. After opening the gigaseal, the microelectrode amplifier (EP-10, HEKA) was set to zero holding current, and cells were stimulated at a frequency of 1 Hz. Recordings were corrected for a liquid junction potential of 11.8 mV[47, 48].

**Chemical screening platform**
A library consisting of FDA-approved drugs (Prestwick, Harvard Medical School, ICCB-Longwood facility) and ion channel library (Selleckem) was used for high throughput screening.

*Zebrafish screening.* We used a previously published approach to automated physiologic screening in the zebrafish exploiting an 'off the shelf' automated microscopy platform (DiscoveryOne) and in house scripts to obtain and analyze high-speed videos from each well in a multiwell plate. Zebrafish were lysed in luciferase buffer afterwards and light intensity associated with nppb expression was quantified for each well. For every criterion a binary score was given to each compound based on its positive or negative effect on the fish. None of the DMSO treated mutant fish were able to attain a score of 3 or 4, while majority of the WTs controls score 4. Compounds scoring 4/4 on the initial zebrafish screening were selected for further testing.

Sulfonylureas, angiotensin receptor blocker, angiotensin converting enzyme inhibitors, beta-blocker, alcohol dehydrogenase inhibitor and calcium channel blockers were among the initial screen hits, each of which has been found to be partially effective in AF management or targets a molecule already implicated in AF pathophysiology[49-54]. Four out of 35 normalized all 4 endpoints (two amlodipine derivatives, praziquantel and carbenoxolone), while 20 compounds normalized only three of four endpoints in subsequent cherry picking experiment. Again, only compounds scoring 4/4 in cherry picking were picked for additional confirmation (Supplementary Figure 5B and Supplementary table 1).

*Cell based screening.* D25 hES-atrial cells derived from MYL4-/- were replated into 384 well plates. After 5 days, cells were treated with the same library of chemicals that was used for zebrafish assay for a total of 7 days. 14 days after starting the treatment, cells were fixed and labeled with an antibody that targeted RDH10. Using a high content imaging system (Harvard Medical School, ICCB longwood screening facility), percentage of the cells positive for RDH10 was calculated for each treatment group. About 20% of vehicle treated D45 MYL4-/- hES-atrial cells were positive for RDH10, whereas only 4% of the D45 MYL4+/+ hES-atrial cells were positive for RDH10. Compounds which resulted in RDH10 percentage fell between 1 SD above/below wild-type and mutant lines, respectively, were selected to narrow-down the zebrafish screening hits that were re-confirmed in the cherry picking process (Supplementary table 1).

## Co Immunoprecipitation (co-IP)

Co-IP was performed according to according to Dynabeads M-280 sheep anti-rabbit kit (Thermo Fisher) manual. Briefly, beads were washed 3x with wash buffer. Next, they were incubated with 2.5% BSA in wash buffer for 1 hour at 4 degrees while on shaker, followed by 2 hours incubation in 2.5% BSA+wash buffer and 2 hours of incubation in 0.5% BSA+wash buffer+MYL4 antibody directed against amino acids upstream to the mutant truncation (Sigma), therefore could pull-down the mutant proteins as well as wild-types (Supplementary Figure 2A).

Cells were grown in 6-well plates and were scraped after 2x PBS wash containing protease and phosphatase inhibitors (Roche). Lysis buffer containing protease and phosphatase inhibitors was added to the pellet after spinning down for 5 minutes 1500 rpm. Cell lysate was spun down for 5 minutes at 12k rpm. Cell lysate was added to the antibody coated beads and kept on a shaker overnight at 4 degrees. Next day, beads were washed 3x with wash buffer and proteins were eluted by adding RIPA buffer to the beads and heating to 72 degrees.

Proteins were loaded at equivalent concentrations and blots were exposed to these antibodies: MYL4 (directed against the full MYL4 amino acid sequence; Proteintech group), Cx43 or Actin.

## Actin polymerization assay

A fluorescence-based assay was used to quantify polymerization (Actin polymerization biochem kit; Cytoskeleton, Inc). Briefly, G-actin solution was prepared by adding pyrene actin to G-buffer, according to kit instructions. G-actin solution was added to appropriate number of wells according to test conditions. Test conditions in our experiment included buffer, recombinant MYL4 protein (Proteintech), VCA+Arp2/3 (Cytoskeleton, Inc), MYL4+VCA+Arp2/3.

## Western Blotting

Protein samples for Western blot were isolated from differentiated cardiomyocytes from one well of a 24-well plate. The membrane and cytoplasmic protein portions were isolated using the Mem-PER□ Plus Membrane Protein Extraction Kit (Thermo Fischer) following Protocol 1: Adherent Mammalian Cells. Equal volume of 2X Laemmli sample buffer was added to the protein lysate and samples were boiled for 5 minutes at 90□C. Samples were run on a 4 – 15% gradient polyacrylamide Mini-PROTEAN□TGX□ Precast Protein Gels (Bio-Rad) at 100V for 100-120 minutes. Protein was transferred to a nitrocellulose membrane and blocked with 1% ECL Prime□ blocking agent (GE life sciences) in tris-buffered saline with 0.1% tween 20 (TBS-T; Boston Bioproducts) for one hour at room temperature. Primary antibodies were incubated at 1:1000-1:2000 in 5% bovine serum albumin in TBS-T overnight at 4□C followed by three 5-minute washes with TBS-T. Secondary antibodies were incubated at a concentration of 1:2000-1:7500 in 5% BSA in TBS-T. After three washes in TBS-T, membranes were developed using ECL□ Prime Western Blotting Detection Reagent (GE life sciences) and visualized using an Invitrogen□ iBright□ FL1000 (Thermo).

## Human atrial tissue samples

Human right atrial appendage tissue samples were harvested as a part of the ongoing prospective CAREBANK study (ClinicalTrials.gov Identifier: NCT03444259). Ethical committee of the Hospital District of Southwest Finland approved the protocol and study complies with the Declaration of Helsinki as revised in 2002. All patients gave their written informed consent prior

to surgery. The study has been enrolling consecutive patients undergoing open-heart cardiac surgery (coronary bypass surgery, operations for valvular heart disease and ascending aorta, and neoplasms of the heart) since February 2016 at Turku University Hospital. Clinical data collection was performed prospectively in an electronic case report form by a trained research nurse with structured questionnaires preoperatively and at pre-specified follow-up times using electronic patient records and phone calls (phone calls at 3, 12 and 24 months postoperatively). Patients were from the catchment areas of Turku University Hospital, Satakunta Central Hospital and Vaasa Central Hospital. These hospitals treat all acute coronary syndromes, revascularizations and cerebrovascular events exclusively in the area. A third party licensed entity monitored the data.

Preoperative 12-lead ECGs were taken one the day prior to surgery and analyzed by a single experienced member of study team for the rhythm, P wave morphology, P wave duration, PTF, the presence of biphasic P waves in inferior leads and PR interval. Echocardiograms were analyzed by an experienced cardiologist.

We included only patients undergoing coronary bypass without any concomitant procedures. Patients with valvular heart disease, ascending aortic aneurysms, prior congestive heart failure, or ejection fraction <45% were excluded. Patients were divided into three groups according to their rhythm: 1) patients always in sinus rhythm preoperatively, during hospitalization and up to two years' postoperative follow-up based on hospital records and phone call follow-ups (SR); 2) patients with atrial cardiomyopathy as indicated by P wave duration >120ms (ACM); 3) patients with preoperative permanent AF (AF).

For immunostaining, all tissue samples were fresh frozen and stored with the patient-specific encryption code at -80 C. Cryosections (6 microm) were performed in OCT blocks.


### Metabolomics analysis

D60 hES-atrial cells (n=4 MYL4+/+, n=5 MYL4-/-) were incubated for 2 hours with fresh medium. This was followed by polar metabolite extraction from cells using cold 80% methanol/water mixture. Next, polar metabolites were isolated and enriched using the protocol developed by Yuan M et al.[55] The metabolite extracts were run using a targeted AB SCIEX 5500 QTRAP LC-MS/MS system via selected reaction monitoring (SRM). The peak areas were subjected to relative quantification analyses with MetaboAnalyst 3.0.[56] To measure intracellular NAD or NADH levels in D60 atrial cells derived from MYL4-/- treated with vehicle, Carb or GAP19, an NAD/NADH quantification kit was used (Sigma-Aldrich).

### Zebrafish cardiac optical mapping and calcium imaging

Optical mapping and signal processing was performed as previously described[57]. For ratiometric calcium recordings, hearts were stained for 15 minutes with FURA-2, AM calcium sensitive dye at 50µM (Invitrogen), and washed with NT-BSA at room temperature for 30-45 minutes to allow complete intracellular hydrolysis of esterified dye. Individual hearts were transferred into perfusion chamber (RC-49MFS, Warner Instruments), which contained NT supplemented with 1 mM Cytochalasin D to inhibit contraction. The chamber was mounted on the stage of an inverted microscope (TW-2000, Nikon). Excitation light was generated by a 120W metal halide lamp (X-Cite 120, Exfo). A high-speed monochromator (Optoscan, Cairn Research Ltd, UK) was used to

rapidly switch the excitation wavelength between 340 nm and 380 nm with a bandwidth of 20 nm at a frequency of 500 s-1. Wavelength switching was well synchronized with the camera to ensure that fluorescence acquisition occurred at well-defined intervals at which the monochromator had reached the two target wavelengths. Each ratio acquisition required four frames, thus resulting in a final ratio rate of 125 s-1. The excitation light was reflected by a 400 nm cutoff dichroic mirror and fluorescence emission was collected by a high-speed 80x80 pixel CCD camera (RedShirtImaging) with 14-bit resolution through a 510/580 nm emission filter. Using a 40x objective and a 0.28 C-mount adapter thus the final magnification was 11.2x resulting in a pixel-to-pixel distance of 2.14 μm. For quantification, images were analyzed with MatLab (MathWorks) using customized software[57]. 3dpf hearts were isolated as discussed above. For voltage recording, individual hearts were loaded with transmembrane-voltage sensitive dye Fluovolt 1x (Invitrogen) for 15 minutes at RT then washed with TS. loaded into a perfusion chamber (RC-49MFS, Warner Instruments), which contained NT supplemented with 1mM Cytochalasin D (Cyto-D; Sigma) to inhibit contraction. The chamber was mounted on the stage of an inverted microscope (TW-2000, Nikon). Fluorescence dye was excited with a 470 nm light emitting diode.

GAP junction hemichannel blockers, CaM kinase and PKA inhibitors were purchased from Tocris (Carbenoxolone cat#3096, GAP19 cat#6227, GAP26 cat#1950 PKI cat#2546, KN 93 phosphate cat#5215). PKC-epsilon inhibitor was purchased from Cayman (Cat#17476). All were used at 5uM concentration in the well unless stated otherwise.

Only atrial measurements were included in the analyses of action potential duration, conduction velocity, calcium amplitude and calcium release duration. Myl4-/- datapoints shown in Figure 3 and 4 are identical for consistency.

**Statistical analyses**
A minimum of 3 biological replicates was performed for each experiment. Error bars in figures represent standard error of the mean and two-tailed student's t-test was performed for analyses unless otherwise indicated in the methods section or figure legends.

**Figure Legends**

**Main Figures.**

**Figure 1. Generation of human atrial cells from pluripotent stem cells. A,** Schematic representation of the differentiation protocol; **B,** Representative image of averaged action potentials recorded from an individual hES-atrial cell. Amplitude 101 mV, Resting potential -81mV and liquid junction potential 11.8mV. **C,** Fluorescence images of the day 60 atrial cells derived from the *MYH6:mCherry* H9 line using the corresponding differentiation protocol. Scale bar =75 μM (MLC2a), 100 μM (cTnT, MYL4 and cardiac actin) and 200 μM (MLC2v). **D,** FACS plot of the day 60 hES-atrial cells sorting based on mCherry expression. **e**, t-SNE plot of single-cell cluster in hES-atrial cells. **F**, Heat map showing key atrial lineage and **G**, differentially expressed markers in top 3 clusters. hES-atrial population. AA: Activin A, B: BMP4, Ch: Chir, RA: Retinoic Acid, Wi: XAV (Wnt inhibitor).

**Figure 2. Single cell RNAseq reveals a distinct cell population in MYL4 mutants. A**, Schematic and **B,** IF analysis for MYL4 in hES-atrial cells showing missing or decreased expression of *MYL4* in the mutant clone. Scale bar =150 µM. **C,** t-SNE plot of single-cell clusters in combined wild-type and mutant populations color coded for discrete clusters (left) and experimental condition (right; KO shown in red vs WT shown in blue). The mutant unique cluster (#0) is circled in red. **D**, Violin plots of top differentially expressed genes between wild-type and mutant lines in cluster #0. **E,** Intracellular FACS staining of cardiac structural markers (Cx43 and alpha actinin) with retinoic acid synthesizing enzymes (ALDH1A2 and RDH10) in day 60 hES-atrial cells from WT, *MYL4*-/- and *MYL4*-/- harboring SNP rs2200733 C-T. **F**, Schematic illustration of generation of *Myl4* mutant zebrafish lines on *Nppb*:Luc background **G,** Immunofluorescent co-staining of ALDH1A2 and S46 (atrial myosin heavy chain) in WT and *Myl4*-/- 5dpf zebrafish hearts. Magnification:30x **H,** Sequencing tracks of open chromatin accessibility for the Rdh10b in 3dpf zebrafish hearts shows distinct ATAC-seq peaks at the promoter region, more frequently in Myl4-/- fish. **I**, t-SNE overlay of selected atrial lineage makers expressed in cardiomyocyte fraction. **J**, violin plots of selected top differentially expressed genes between wild-type and mutant lines in cardiomyocyte population. **K**, Fluorescence actin polymerization assay in the presence of either buffer, MYL4 recombinant protein or ARP2/3+VCA. **L**, Schematic diagram showing patients enrolled in this study for obtaining tissue biopsy that included individuals with normal sinus rhythm, atrial cardiomyopathy (CMP) defined as PR interval>120ms and permanent AF. **M**, Immunoflourescence staining and **N**, quantification of biopsy samples for Phalloidin (marks F-Actin;grey), and MYL4 (red; merged staining shown here; magnification 40x). A higher magnification is shown in the right column. $*p<0.05$, $**p<0.01$, $*** p<0.001$.

**Figure 3. A highthroughput combined *in vivo* and *in vitro* screening platform to identify disease modifiers**

**A**, cardiac function quantification of myl4 mutant zebrafish lines on Nppb: luc background (left) and the schematic illustration of in vivo screening platform design (right). *p<0.05, **p<0.01, *** p<0.001, t-test. Data are mean □ s.e.m. **B**, %RDH10 in day 45 hES-atrial cells derived from wild-type vs mutant lines (right) and schematic representation of the cell based assay (right). ****p<0.0001,t-test. Data is presented as mean □ s.e.m.  **C, D** Results of primary screening of 1280 FDA approved chemicals for (c) zebrafish heart rate, contractility, tail blood flow (56 hpf), luciferase level (72 hpf) and (d) %RDH10 in Day 60 hES-atrial cells. Red boxes show the compounds that could met the individual criteria but only the one that fulfilled all the criteria was selected for further confirmation. **E,** Chemical structure of Carbenoxolone, a connexin 43 hemichannel blocker. BF: blood flow, Cont: contractility, HR: heart rate, Luc: luciferase, HT CV: Highthroughout cardiovascular, IF: Immunoflourescence.

**Figure 4. Connexin 43 HC blocker prevents membrane leak and reverses electrophysiological changes in atrial fibrillation A**, Immunofluorescent co-staining of Cx43 with MYL4 in D60 hES atrial cells derived from WT and mutant lines. Scale bar =50 µM. **B**, Immunofluorescent co-staining of Cx43 with S46 in 5dpf *Myl4*+/+ and *Myl4*+/- fish lines. Magnification:30x **C,** Immunoglobulin pulldown of MYL4 in D60 *MYL4*+/+ and MYL4-/- hES-atrial cells and Western Blot analysis of MYL4, Cx43 and Actin. Dashed arrow denotes the band corresponding to the MYL4 protein. **D,** Immunofluorescence images and **E**, quantification of Cx43 (green) co-localization with intracellular actin (red; higher magnification in the right column), and the length ratio of Cx43+ and ZO-1+ regions in biopsy samples from individuals with normal sinus rhythm (SR), atrial cardiomyopathy (CMP) and AF. *$p<0.05$, **$p<0.01$, *** $p<0.001$ **F,** Polar metabolite abundance analysis from D60 WT and mutant hES-atrial cells as determined by LC-MS/MS. *$p<0.05$, t-test. Data are mean □ s.e.m. **G,H,** Relative NAD level in *MYL4*-/- D60 hES-atrial cells treated with vehicle, carb (5uM) or GAP19 (5uM). **I,** Absolute action potential voltage amplitude and dv/dt in D60 hES-atrial cells derived from *MYL4*+/+, *MYL4*-/- and *MYL4*-/- after 3 days of treatment with carb (5uM) in a current clamp setting. *$p<0.05$, **$p<0.01$, t-test. Data are mean □ s.e.m. **J,** Averaged APD and j, Calcium amplitude recordings from atrium of 3dpf WT or mutant fish (with or without 2-day drug pretreatment). *$p<0.05$, **$p<0.01$, t-test. Data are mean □ s.e.m. Carb: Carbenoxolone, G19: GAP19, G26: GAP26.

**Figure 5. RA inducible PKC activity leads to Cx43 hemichannel phosphorylation and subsequently electrophysiological changes in AF. A,** Gene ontology analysis of WT vs mutant transcriptional profiling in mCherry+ cells purified from D60 hES-atrial cells. **B,** Phospho-Cx43 fraction in membrane assessed by Western Blot in MYL4 mutant vs WT hES-atrial cells **C,** with increased underlying scaffold stiffness and **D,** mutants harboring rs2200733 C-T risk allele. **E,** Schematic representation of Cx43 c-term phosphorylation sites. Average **F,** APD and **G,** Calcium transient amplitude from atrium of 3dpf WT or mutant fish (with or without 2-day drug pretreatment) heart fluorescence after incubation with cell membrane voltage dye or calcium dye, respectively. *$p<0.05$, t-test. Data are mean □ s.e.m. **H,** Phospho-Cx43 fraction in membrane assessed by Western Blot in MYL4 mutant treated with PKC inhibitor, PKA inhibitor and RA (1uM and 0.1uM) with or without AGN193109. **I,** Relative NAD level in *MYL4*-/- D60 hES-atrial cells treated with vehicle, PKCi (5uM) or CAMK2i (5uM) **J,** PKC activity measured in *MYL4*-/- derived D60 hES-atrial cells treated with vehicle, or RA or RA+AGN193109. **K,** A summary of changes at the cellular level in a subset of the AF models studied here. Carb: Carbenoxolone, G19: GAP19, G26: GAP26.

**Supplementary Figures.**

**Supplementary Figure 1. Generation of a reporter system for characterization of hES-atrial cells derived from hESCs and in depth characterization of human atrial lineages at single cell level  A,** Schematic representation of dual reporter line; **B**, IF images of hESC-derived atrial cells for mCherry:MYH6, co-stained for cTnT, Myosin Heavy chain and cardiac actin. Scale bar =100. **C**, FACS plot of hES-derived atrial cells for the percentage positive of GFP expression. **D**, FACS plot of H9 hES-derived atrial cells used for gating the mCherry population in MYH6:mCherry reporter line. **E**, Heat map showing key differentially expressed markers in clusters 3-7 and **F,** the atrial/ventricular, conduction system and epicardial lineage markers.

**Supplementary Figure 2. Characterization of MYL4 frameshift mutants in human pluripotent stem. A** Sequencing result of human mutant lines aligned with Refseq. **B**, Western blot analysis of MYL4 protein by an antibody directed against the full amino acid sequence (left) vs against the sequence upstream to frameshift mutation (right).

**Supplementary Figure 3. Upregulation of retinoic acid synthesis pathway and response elements in MYL4 mutants lines and also in cell lines harboring the PITX2c risk allele.**

**tSNE and violin plots for key atrial and differentially expressed markers in combined wild-type and mutant population. A,** tSNE plot with cluster numbers **b,** tSNE plots for atrial lineage markers. **B,** Violin and **C,** tSNE plots for the top genes expressed in circled cluster (related to Figure 2a). **D,** Sequencing result (upper panel) and Western blot analysis of PITX2 (lower panel) in human MYL4-/- line harboring rs2200733 risk allele.

**E,** Sequencing result of zebrafish mutant line aligned with Refseq. **F, G** Open chromatin accessibility analysis of 3 dpf zebrafish heart. **H,** ATAC-seq profiles across whole genome of wild-type and mutant fish.

**Supplementary Figure 4. Top differentially expressed genes between wild-type and mutant lines in clusters expressing MYH6.**

**Supplementary Figure 5. Immunoflourescence staining of human heart biopsies for MYL4 and F-Actin.**

Heart biopsies obtained from individuals with normal sinus rhythm, atrial cardiomyopathy (CMP) defined as PR interval>120ms and permanent AF enrolled in this study were stained for MYL4 (red), phalloidin (grey) and DAPI (blue; magnification 40x).

**Supplementary Figure 6. An in vivo and in vitro small molecule screening platform to identify pathways involved in AF substrate pathology. A,** Original traces of sequencing tracks of open chromatin accessibility for the *Nppb* locus in 3dpf zebrafish hearts shows distinct ATAC-seq peaks at the promoter region in wilt-type fish but not mutant lines.

**B**, Homozygous mutants were in-crossed and after 24 hours, were re-distributed into separate wells (3 embryos/well) of a 96-well plate and subsequently were treated with a unique compound. Last column of each 96well plate consisted of only WT fish. First and last column was treated with DMSO. Using a motorized XYZ microscope, a 3sec video was recorded from each quadrant of the plate at 56 hpf. Individual fish were localized based on detection of their heart beat and tail flow. According to these landmarks, heart rate, contractility and tail flow were quantified. At 72hpf, luciferase lysis buffer was added to the same plate and luciferase intensity was measured for each well. **C**, Cherry picking result of primary 36 hits **D**, dose curve analysis of Carbenoxolone.

**Supplementary Figure 7. Cx43 trafficking changes in disease conditions.**

**A**. Electron microscopy images of 7dpf zebrafish WT and *Myl4* mutant atrium tissue. Note unchanged myofibrillar array between WT and mutant (white arrow) and dense granules at the inter-cellular junction in mutants (black arrows). Scale bar = 500 nM. **B,** Full WB images related to Figure 3c. Arrowhead, solid arrow and dashed arrow denote the bands corresponding to the heavy chain, light chain and MYL4 protein, respectively. **C,** Heart biopsies were obtained from individuals with normal sinus rhythm, atrial cardiomyopathy defined as PR interval>120ms and permanent AF. Samples were stained for Phalloidin (marks F-Actin), Cx43, and DAPI. Magnification 40x.

**Supplementary Figure 8. Metabolomics and electrophysiological analysis of genetically engineered hES atrial cells and zebrafish. A,** Polar metabolite abundance analysis from D60 WT and mutant hES-atrial cells as determined by LC-MS/MS. *p<0.05, ***p<0.0001, t-test. Data are mean $\Box$ s.e.m. **B,** Maximum diastolic potential in D60 hES-atrial cells derived from *MYL4*+/+, *MYL4*-/- and *MYL4*-/- after 3 days of treatment with carbenoxelone in a current clamp setting.

**Supplementary Figure 9. Voltage and calcium mapping recordings from atrium of 3dpf WT or mutant fish. A,** Averaged conduction velocities from atrium of 3dpf WT or mutant fish (with or without 2-day drug pretreatment) heart fluorescence after incubation with cell membrane voltage dye. Data are mean ☐ s.e.m. **B, C** Averaged Ca release duration from atrium of 3dpf WT or mutant fish (with or without 2-day drug pretreatment) heart fluorescence after incubation with Fura-2. Data are mean ☐ s.e.m. Carb: Carbenoxolone, G19: GAP19, G26: GAP26. **D,** Full Western blot image of Cx43 expression in MYL4-/- hES-atrial cells treated with control vehicle vs different drugs (related to Figure 4h).

Supplementary Table 1. List of hit compounds identified in combined in vivo and in vitro screening.

| | First round fish score | Second round fish score | hES-atrial RDH% correction |
|---|---|---|---|
| Glipizide | 4 | 2 | |
| Tolazamide | 4 | 2 | |
| Tizanidine | 4 | 2 | |
| Fomepizole | 4 | 2 | |
| Dicloxacillin | 4 | 3 | |
| Sertindole | 4 | 2 | + |
| Sulmazole | 4 | 1 | + |
| Suxibuzone | 4 | 3 | |
| Methotrimeprazine | 4 | 2 | |
| Carbinoxamine | 4 | 3 | |
| Trimipramine | 4 | 3 | |
| Carbenoxolone | 4 | 4 | + |
| Nadolol | 4 | 3 | + |
| Ganciclovir | 4 | 3 | |
| Lorglumide | 4 | 2 | |
| Altretamine | 4 | 3 | + |
| Oxybenzone | 4 | 1 | + |
| Pramipexole | 4 | 3 | + |
| Piretanide | 4 | 1 | + |
| Gatifloxacin | 4 | 3 | |
| Telmisartan | 4 | 2 | + |
| Phentermine | 4 | 3 | |
| Fosinopril | 4 | 2 | + |
| Flumethasone | 4 | 2 | |

| Azatadine | 4 | 2 | + |
|---|---|---|---|
| Amlodipine besylate | 4 | 4 | |
| Amlodipine | 4 | 4 | |
| Procaine | 4 | 3 | |
| Diazoxide | 4 | 3 | |
| Mianserine | 4 | 3 | + |
| Bupropion | 4 | 3 | + |
| Ethacrynic acid | 4 | 3 | |
| Clomipramine | 4 | 2 | |
| Methylprednisolone | 4 | 3 | + |
| Praziquantel | 4 | 4 | |

Supplementary Table 2. List of antibodies used for immunoflourescen, western blot and FACS analysis.

| Item | Vendor | Concentration |
|---|---|---|
| Connexin-43 antibody | Sigma | 1:400 (1:2000 for WB) |
| RDH10 Antibody | Proteintech | 1:400 |
| ALDH1A2 antibody | Sigma | 1:400 |
| MYL4 antibody | Sigma<br>Proteintech | 1:250 (1:2000 for WB)<br><br>1:400 (1:2000 for WB) |
| PITX2 antibody | Proteintech | 1:1000 (WB) |
| cTnT antibody | Thermo Fischer | 1:500 |
| MLC2a antibody | Synaptic Systems | 1:200 |
| MLC2v antibody | Synaptic Systems | 1:200 |
| Cardiac actin antibody | Sigma | 1:400 |
| S46 antibody | DSHB | 1:200 |
| MF20 antibody | DSHB | 1:200 |

| | | |
|---|---|---|
| Beta-actin | Cell signaling | 1:2000 (WB) |
| ZO-1 | Millipore | 1:200 |
| Alexa Fluor 647 phalloidin | Invitrogen | 1:200 |

Supplementary Table 3. List of primers and gRNAs used for sanger sequencing and generation of genetically engineered lines.

| Name | Sequence |
|---|---|
| Human *MYL4*-gRNA1 | TTGTTTGACCGGACCCCGACTGG |
| Human MYL4-gRNA2 | CCCGCAGTACATCCCCGCACTGG |
| Danio Rerio *Myl4_exon3*-gRNA1 | AGATCACTTATGCCCAGTGTGGG |
| Danio Rerio *Myl4_exon3*-gRNA2 | ATTTCTCTCACCCTCAGGTCTGG |
| Danio Rerio *Cmlc1*-gRNA1 | GCTTGGGGAGAAAATGACAGAGG |
| Danio Rerio *Cmlc1*-gRNA2 | CGTGTAGTTAATGCAGCCGTTGG |
| Human *MYL4*-F<br>Human *MYL4*-R | TTGACAGAAGATGGGATGAGGACGG<br>ACACACTCCCTCACACCTCTTCTCATCTG |
| Danio Rerio *Myl4*- F<br>Danio Rerio *Myl4*- R | GCTAATCCCACAATGACGGTTTAGG<br>CAAAGTCCTCAAAAGTGCCCTGATC |
| Danio Rerio *cmlc1*- F<br>Danio Rerio *cmlc1*- R | AGCCCAGACCAGATTGAAGAGTTCAG<br>TTCCAACTCCACACACATCTAAGTTTGAC |
| PITX2-rs2200733 locus gRNA | TGATCAGAGAAAATTAGAAC |
| Human *PITX2*-F<br>Human *PITX2*-R | CTTGCATGTACGACTCCCTTTCTGAC<br>TGACCCAAAATAGGTAAGGAGCCTAGAG |
| SHOX2 gRNA | GGCGTTGGCGTCACAGACCC |
| MYH6 gRNA | GCAGCAAAAAATGCACGATG |

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