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Genetic varients leading to atrial fibrillation

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SCHOOL OF MEDICINE

Thesis

GENETIC VARIANTS LEADING TO ATRIAL FIBRILLATION

by

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GENETIC VARIENTS LEADING TO ATRIAL FIBRILLATION

ELIZABETH ABRAHAM

ABSTRACT

Background

Atrial Fibrillation (AF) is the most common cardiac arrhythmia, affecting over 3 million Americans. Many people who suffer from AF have pre-disposing factors such as hypertension, ischemia, and structural heart disease, but recent research has also demonstrated the importance of genetic factors that can contribute to AF. In the present study, we sought to determine the causative mutation in a family with AF, atrial septal, and ventricular septal defects.

Methods

We evaluated a pedigree with 16 family members, one of whom had an ASD, one a VSD, and three had AF. Exome sequencing was performed on three of the five affected family members followed by confirmation with Sanger sequencing in all family members. A separate cohort from the MGH AF Study with early-onset AF (age at onset 47.1 ± 10.9 years, 79.3% male) was also screened for mutations using a combination of Sanger sequencing and high resolution melting. Variants were then functionally characterized using reporter assays in a mammalian cell line using wild-type and mutant constructs driving *NPPA*, *αMHC* and *NPPB* promoter reporters.

Results

Exome sequencing of the three affected individuals in the family identified a highly conserved mutation, R585L, in the transcription factor gene, *GATA6*. We also identified three additional *GATA6* variants (P91S, A177T, and A543G) in the cases with early-onset AF from the MGH AF Study. We found that three of the four variants had a marked upregulation of luciferase activity (R585L; 4.1 fold, $p<0.0001$; P91S; 2.5 fold, $p=0.0002$; A177T; 1.7 fold, $p=0.03$). Additionally, when co-overexpressed with *GATA4* and *MEF2C*, all *GATA6* variants exhibited upregulation of the *αMHC* and NPPA activity compared to control.

Conclusion

Overall, we found gain-of-function mutations in *GATA6* in both a family with early-onset AF and atrioventricular septal defects as well as in patients with sporadic, early-onset AF. This evidence suggests that specific gain of function mutations in *GATA6* contribute to the development of AF.

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LIST OF ABBREVIATIONS

AAD…….Anti-arrhythmic drug

Alpha-MHC……..Alpha subunit of myosin heavy chain

AF……..Atrial Fibrillation

ANP……..Atrial naturetic peptide

ASD……Atrial Septal Defect

CHO…….….Chinese hamster ovary cells

COS7…….African green monkey kidney fibroblasts

ECG……..Electrocardiogram

EGFP…….....……Enhanced green fluorescent protein

GAPDH……...…Glyceraldehyde-3-phosphate dehydrogenase

GATA4……GATA binding factor 4

GATA6…….…GATA binding factor 6

GWAS……Genome Wide Association Study

HEK293…….……..Human embryonic kidney cells

HHAT……...……Hedgehog acyltransferase

HHATL……...……Hedgehog acyltransferase-like

HRP…….……Horseradish peroxidase

KCNN3.....Potassium channel calcium activated subfamily N alpha member 3

KCNQ1…….……Potassium voltage gated subfamily Q member 1

MEF2C……....Myocyte specific enhancer factor 2C

NKX2.5…….NK2 homeobox 5

NPPA……...…….Nautriuretuc peptide A

NPPB……...…….…….Nautriuretuc peptide B

PBS…….....……Phosphate Buffered Saline

PCR……..…Polymerase chain reaction

PITX2……...….Paired-like homeodomain 2

RT-PCR……..…..Reverse transcription polymerase chain reaction

SDS-PAGE……..Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Shh……....……Sonic hedgehog

Shh-Np…….Sonic hedgehog pamitoylated at the N-terminus

SNP…….....…Single Nucleotide Polymorphism

SNAP……SNP annotation and proxy search

TBX5…….……T-box transcription factor 5

VSD……...…Ventricular Septal Defect

ZFHX3……Zinc finger homeobox 3

INTRODUCTION

Atrial Fibrillation (AF) is the most common heart arrhythmia affecting about 5.2 million in the United States and nearly 30 million people worldwide (Chugh et al., 2014). The number of cases in the U.S. alone is expected to double by 2030 (Colilla et al., 2013), making research into the etiology and causes of AF a necessity. AF affects the electrical functioning of the atria or upper chambers of the heart and can be described as an irregular, rapid heartbeat. Although AF is often considered a minor issue, it commonly causes blood clots, stroke, myocardial infarction, dementia and heart failure (Mozaffarian et al., 2016). In the Framingham Heart Study, a nearly five-fold increased incidence of stroke was found in patients with AF in the absence of coronary heart disease and heart failure. When concomitant with heart failure and coronary heart disease AF further increases ones risk of stroke as compared to individuals with coronary heart failure and heart failure alone (Wolf, Abbott, & Kannel, 1991). Additionally, individuals with AF have about twice the risk of death as compared to the general population (Benjamin et al., 1998).

I. Pathophysiology of AF

Physiologically, AF reduces cardiac output thus decreasing blood flow to the brain and other organs. There is no known cure for this disease and current treatment options are limited, making research into its mechanisms and pathophysiology paramount (Prystowsky, Padanilam B.J., & Waldo A.L., n.d.). AF typically originates from a rapidly firing ectopic loci within the proximal pulmonary veins and an abnormal atrial tissue that maintains this arrhythmia by allowing it to propagate via re-entrant loops. Chronic AF is

also capable of changing overall atrial electrophysiology by causing refractory period shortening, which further perpetuates the arrhythmia in a vicious cycle. The majority of people who suffer from AF have pre-disposing factors that increase their risk of developing the disease such as, hypertension, ischemia, and/or structural heart disease (Markides & Schilling, 2003). Other risk factors include: age, sex, valvular heart disease, cardiomyopathy, diabetes mellitus, obesity, sleep apnea, chronic kidney disease, thyroid dysfunction, smoking, and alcohol consumption (Andrade, Khairy, Dobrev, & Nattel, 2014).

Atrial and ventricular septal defects are common congenital structural heart defects that can often lead to AF, even after surgical treatment. Both are holes in the heart wall (separating the two atria and ventricles respectively) that fail to close properly during development. The severity of these defects can vary from almost undetectable to life-threatening. Congenital heart disease is the most common birth defect affecting 1-2% of newborns. The incidence of arrhythmias increases as the individual with a heart defect ages (Khairy & Balaji, 2009).

II. Early Genetic Studies

AF is often secondary to other conditions however, up to 30% of patients have lone, or idiopathic AF (Parvez & Darbar, 2011). Recently, researchers have discovered a number of genetic factors that are known to contribute to AF, especially in cases in which first degree relatives are known to have the disease (Steffensen et al., 2015).

The first study of related families with early onset AF to establish that there is a genetic basis for the disease was in 1997 (Brugada et al.). This study screened a 26-

member family, 10 of which had AF, and performed linkage analysis and found that their disease was linked to a single locus. They also found similar results in two separate families. Soon after, Chen et al. (2003) discovered the first genetic mutation causing AF in the potassium voltage gated subfamily Q member 1 gene (*KCNQ1).* The study examined the gene sequences of a family with hereditary persistent AF and found a mutation in a *KCNQ1* gene that was not present in normal individuals. This research has spurred much investigation into the genetic basis for AF.

III. Heritability of AF

Familial studies give us insight to rare variants with a very high penetrance (such as *KCNQ1*). Meaning, the chances of developing AF increase dramatically if you have one of these variants. Based on data from the Framingham Heart Study a person's chance of developing AF increases about 40% with one immediate family member having the disease (Figure 1) (Lubitz SA, Yin X, Fontes JD, & et al, 2010). Whereas if one has a variant within a locus found in one of the genome wide association studies (GWAS, described below), that person's chance of developing AF is not quite as high (approximately a 10% increased risk) (Figure 2). This is because GWAS variants are more common and are not typically inherited.

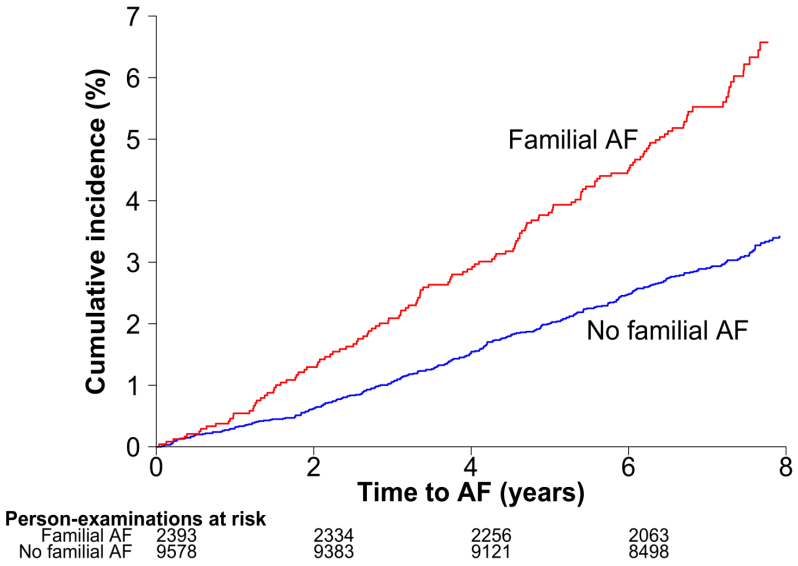

Figure 1. AF in first degree relatives associated with an increased risk of AF, (Lubitz et al 2010).

Heritability may also play a role in nonfamililal atrial fibrillation. Christophersen et al. studied Danish monozygotic (identical) and dizygotic (fraternal) twins and examined their risk for developing AF if their co-twin has the disease. They found that concordance rates for identical twins were two times greater than for fraternal twins (2009). Because identical twins have the same genome this study suggests that the variation in the concordance rates between identical and fraternal twins is entirely dependent on their genes.

A similar study sought to determine heritability of AF in Icelanders. Icelanders are a relatively homogeneous population in terms of both genetic and environmental factors. This study found that first degree relatives of AF patients were more than four times more likely to develop AF and also that second degree relatives of AF patients had a higher risk of developing the disease than the general population. This is significant

because it ascribes more weight to the genetic basis of AF and rather than the home environment (Arnar et al., 2006).

The MGH lone AF registry has also been examined along with their families which found that over one third of these patients had evidence of familial disease. They also validated that members of patient's families with lone AF have a much higher prevalence of AF as compared to the general population. The family members of probands with lone AF have a significantly increased risk of AF compared to that of the general population. The overall sibling recurrence risk ratio was 57 (Ellinor, Yoerger, Ruskin, & MacRae, 2005). This large relative risk to siblings is strongly suggestive of a Mendelian mode of inheritance.

IV. GWAS Studies

Due to technological advances in genotyping, many studies have performed GWAS using much larger cohorts of patients to identify different susceptibility loci. These large cohorts compare participants suffering from AF against those who do not suffer from the disease to find genetic factors that differ between them. The first GWAS was performed in an Icelandic population and lead to the discovery of a susceptibility locus near the Paired-like homeodomain 2 gene (*PITX2*) that was later also replicated in European and Asian populations (Gudbjartsson et al., 2007). In 2009, another locus was identified by two separate groups within the Zinc finger homeobox 3 gene (*ZFHX3*- which also encodes a transcription factor) in Europeans and Han Chinese populations (Gudbjartsson et al., 2009) (Benjamin et al., 2009). Another one of the earliest GWAS was conducted using 1,335 cases of lone AF and 12,844 control patients of European

decent. This GWAS study uncovered another common AF risk locus, *KCNN3*, a calcium activated potassium channel (Ellinor et al., 2010).

In 2012 a GWAS meta-analysis compared 6,000 individuals with AF to over 50,000 without and identified 6 additional variants. Recently, many new loci were explored by Sinner et al. in a GWAS cross ethnic study, which took this idea further by comparing completely different cultures. Sinner et al. identified 5 novel loci for AF in two different populations of Europeans and Japanese patients with AF (2014). Very recently, a much larger consortium of 19,000 AF patients was utilized to identify 7 additional loci with a much greater power (Ellinor, unpublished).

In sum, there are currently 23 AF susceptibility loci known that have been identified using GWAS. Of these loci, five are known transcription factors six are ion channels and related proteins, three are known myocyte proteins, and the remaining nine have an unclear relation to AF. These studies show that there are many genes that can contribute to AF. Although their exact mechanisms of action and interplay remain unclear, these studies provide direction for future investigation of the molecular pathways indicated.

Of note, in current AF GWAS studies the most strongly associated locus is near *PITX2*. *PITX2* is a transcription factor for which a more defined mechanism exists (A Palatinus & Das, 2015). *PITX2* deletion studies have shown an increase in gene expression in genes specific to the sinoatrial (SA) node. Additionally, *PITX2* has been shown to down-regulate the expression of Nppa (nautriuretuc peptide A-which regulates intravascular volume) and *KCNQ1* (encodes a potassium channel). Variants in Nppa and

KCNQ1 have both been linked to cases of familial AF and have caused atrial action potential (AP) shortening and changes in calcium currents (Zhou, Liao, & Tu, 2015).

V. Missing Heritability of AF

GWAS studies help us identify common variants within populations that have AF while familial studies add another important piece to this puzzle; namely, rare variants with high penetrance. Still, there is a substantial amount of grey area in-between these two extremes and a gap between the current number of variants found to date and the population attributed risk (Magnani et al., 2011). Overall, the risks associated with common variants found to date statistically only explain 10% of the heritability of AF (Parvez & Darbar, 2011).

This missing heritability could be due to common variants that have not been found by GWAS, epigenetics, rare variants with strong effects, large or small copy number variants, and mosaicism (when the genomic content is not uniform across all cells, mainly due to errors in cell division) which is difficult to assess with current techniques (Tucker, Clauss, & Ellinor, 2016). These errors include single nucleotide substitutions, copy number alterations, rearrangement, and chromosome gains and losses. Mosaicism is often recognized as being responsible for germline disorders (for example, nondisjunction in trisomy 21) but it can also occur within somatic cells in which case these errors can accumulate over time (De, 2011).

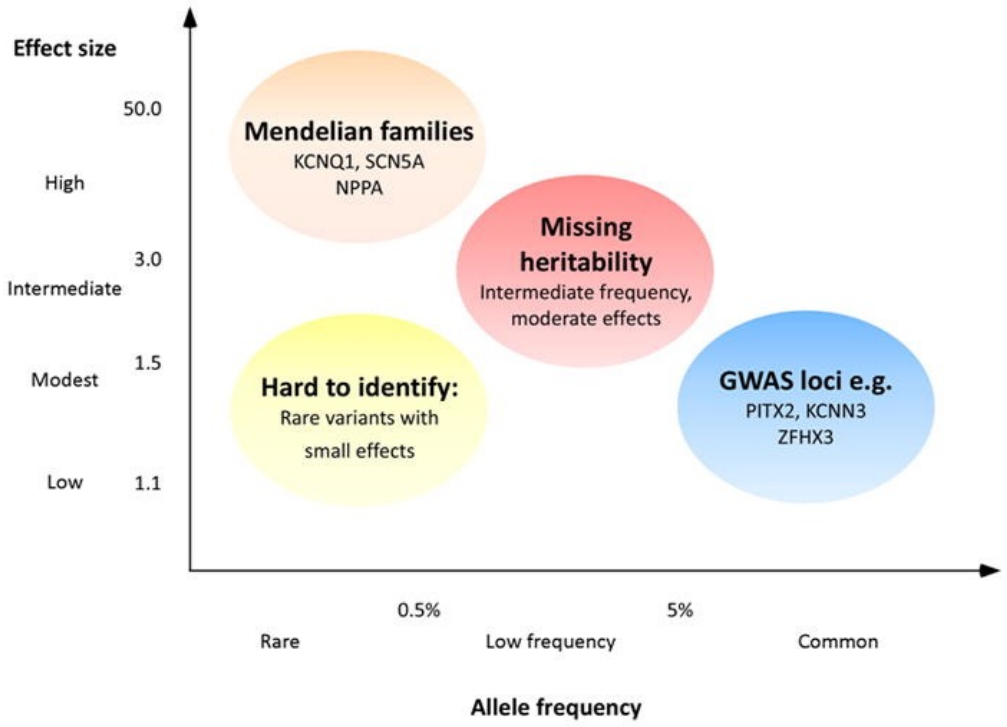

Figure 2. Frequency of known AF genetic variants versus effect size, (Magnani et al., 2011).

While mosaicism and lower frequency variants with intermediate effect sizes are harder to detect, it is hoped that much of this missing genetic component will be discovered as further advances in sequencing technology (such as whole-genome sequencing) become more readily available.

VI. Treatments of AF: Present and Future

In clinic AF is typically initially treated with beta-blockers and blood thinners. Beta-blockers are used to slow the overall heart rate to prevent the rapid firing from the atria from propagating to the ventricles while blood thinners are used to prevent stroke. If a patients AF progresses to severe and symptomatic antiarrhythmic drug (AAD) therapy may be attempted. The most common antiarrhythmic used is Amiodarone, which prolongs the cardiac action potential mainly by blocking sodium and potassium channels. A major downside of these antiarrhythmic drugs is that they have high toxicities and can actually be proarrythmic at certain doses. Also, over half of patients treated with antiarrhythmic drugs experience a reoccurrence of AF within one year (Hohnloser, Kuck, & Lilienthal, 2000). If antiarrhythmic drug therapy is unsuccessful catheter based ablation can be performed. The procedure burns atrial tissue (usually around the pulmonary veins) to electrically isolate that arrhythmogenic area to stop it from spreading. This is can be a very effective procedure initially, however, over time AF likely to reoccur especially in older patients (Bunch et al., 2016).

Overall, there is a lack of effective therapies for AF. This, in part, is due to an incomplete understanding of the complex pathophysiology of AF. The important outcome of genetic studies is that they can further elucidate the potential molecular mechanisms and find potential targets for therapy. Currently, AF response to treatment is highly variable among patients. This is thought to be partially due to slight differences in disease mechanisms between individuals. Some of these mechanisms include changes in the atrial action-potential duration, conduction slowing, weakened cell-to-cell

communication, modulation of signaling proteins, and atrial fibrosis and inflammation (Darbar & Roden, 2013).

An SNP at the 4q25 locus (near *PITX2*) was shown to be significantly associated with successful symptom control with AAD. Also, these individuals who carried the 4q25 SNP responded better to class I versus class III AADs (Parvez et al., 2012). The same group also found that the same locus near *PITX2* was associated with AF reoccurrence after patients were treated by catheter ablation (Shoemaker et al., 2015). This further implicates that genetics can give us important information on how certain patients should be treated and how different subtypes of AF may exist.

In this era of "personalized medicine" the field of pharmacogenetics is rapidly expanding. It is hoped that within the next five to ten years a more personalized approach will be taken to treat AF and that new drugs will be in development based on the targets identified in these genetic studies.

VII. Current Study

In the present study we sought to elucidate more of the far left of Figure 2. Namely, the rare variation that is highly penetrant. These are typically familial studies aimed at finding the causative mutation in families with hereditary patterns of disease (i.e. diseases with germline inheritance). The family that this study evaluates has a hereditary pattern of early-onset AF and atrial and ventricular septal defects (ASD and VSD respectively).

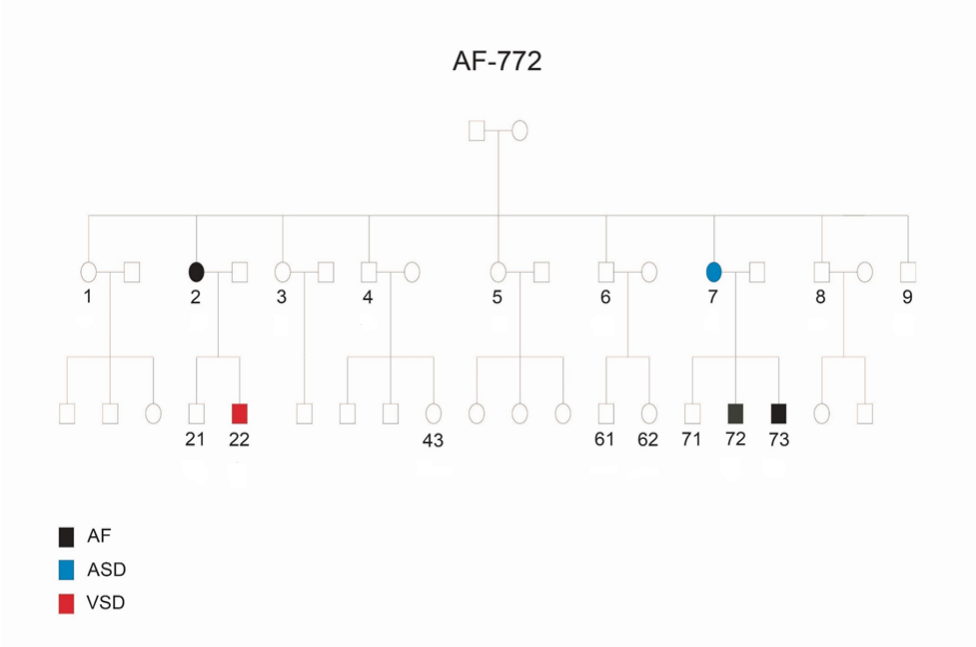

Figure 3. Phenotypic breakdown of family AF-772 (Ellinor, unpublished).

Individual 7, the proband in the family, initially presented with shortness of breath, palpitations and two episodes of near syncope. Subsequent investigations revealed the presence of an ASD. She underwent surgery to correct an ostium primum ASD. Two of her descendants, 71 and 72, were diagnosed with AF at the ages of 14 and 16 years respectively. The proband´s sister, 2, had a single documented paroxysm of AF. The proband´s nephew, 22, had a VSD during childhood.

The trait appears to be inherited in an autosomal dominant patteren with five members of the 16-member family having the disease. Three affected members of the family (22, 72, and 73) were exome sequenced and evaluated for genetic mutations.

Specific Aims and Objectives

Because of the risks associated with AF and its widespread prevalence, effective treatment and prevention of the disorder is crucial. The study of the genetics involved can help expand our understanding of the molecular pathways to enable us to better clarify clinical implications and potential treatments for the disease. This study aims to identify and further elucidate the role of a genetic mutation found in a family with a hereditary AF and atrial and ventricular septal defects (ASD and VSD respectively).

The specific aims of the study are:

1. Identify the causative mutation in family AF-772
2. Determine if variants in this gene could be present in other patients with early onset AF.
3. Describe a functional assay characterizing two potential variants

Different genetic polymorphisms associated with AF manifest themselves differently. This is important in assessing different therapeutic approaches, as different methods may be more effective in different patient populations depending on their genetics. Through this study we hope to develop a more comprehensive knowledge of the genetic causes for AF with the intention of contributing to different clinical interventions to help decrease the morbidity of this disease.

METHODS

I. Clinical Evaluation of AF-772

Sixteen members of the family underwent a standardized interview, physical examination, 12-lead electrocardiogram and echocardiography. Written informed consent was obtained from all patients prior to performing the evaluations. The study was approved by the Institutional Review Board at Massachusetts General Hospital (MGH) and complied with the Declaration of Helsinki. Affected individuals were identified as those with either AF on the electrocardiogram or patients with a documented ASD or VSD during echocardiographic analysis.

II. Exome Sequencing of Family

Previously our lab exome sequenced 3 members (22, 72, and 73) of a 16 member family (AF-772) with a complex phenotype of hereditary AF, ASDs, and a VSD (Figure 3) using Agilent Sure Select Target Enrichment Assay (version 3) to isolate the exonic regions of their DNA and the Illumina HiSeq 2000 platform at Perkin Elmer (Waltham, MA, USA) for sequencing.

Next, the short reads were aligned to a reference human genome (NCBI Build 37, hg19), in order to identify variants within the exons.

III. Filtration and Prioritization

Filtration and prioritization of the variants was performed according to previously published methods (Li, Gui, Kwan, Bao, & Sham, 2012). Synonymous variants and common variants (>0.01%, identified in publically available databases dbSNP, 1000 Genomes Project (1000 Genomes Project Consortium et al., 2010), and Exome Variant

Server) were filtered out. Common variants are unlikely to be the disease causing mutation in instances of Mendelian disease because if a common variant was responsible for such a penetrant phenotype, one would see far more individuals in the general population with this disease. Additionally, variants that individuals 22, 72, and 73, did not have in common were also excluded from the analysis.

The effect of the variants on protein coding was determined with Ref Gene from the University California, Santa Cruz (UCSC) (i.e. determining synonymous variants, or variants that do not change the amino acid). Prioritization of these variants was based on conservation of the amino acid among species using the Phylop tool, the predicted effect of the amino acid change on protein structure using the Poly-Phen-2 and SNAP tools, and their respective gene expression levels in the heart using the GNF Expression Atlas 2 data from U133A and GNF1H chips in UCSC browser.

Sanger sequencing was used to confirm the presence of the variants and to perform segregation analysis for each individual of AF-772. Oligonucleotide primers flanking the putative variants were designed using cDNA and genomic sequences from the UCSC Genome Browser (hg19 assembly). Standard conditions were used to perform PCR. DNA sequencing was performed using the ABI PRISM dye terminator method (Applied Biosystems, CA, USA).

IV. High Resolution Melting

To determine if the GATA binding factor 6 (*GATA6)* variant found in AF-772 was present within the general population, we screened a cohort of patients from MGH with early onset AF using a combination of high resolution melting and Sanger sequencing.

Exons 3, 5 and 6 of the *GATA6* gene were screened using high resolution melting whereas the remaining coding exons (2, 4 and 7) were sequenced directly using Sanger sequencing. Light Scanner technology (Idaho Technologies Inc. UT) was used to perform high resolution melting according to manufacturer's recommendations. High resolution melting utilizes the fact that the melting temperature of DNA is entirely dependent on its sequence. Therefore, when screening a number of patient samples at once, variants in an individual's sequence can be picked up by comparing their melt curves (using a fluorescent intercalating dye) to the other samples.

V. Functional Assay of GATA6

To determine the functional effects of the *GATA6* variants our lab performed luciferase assays to measure wild-type *GATA6* and our mutant construct's transcriptional activity.

The mammalian expression vector pCMV6 XL6 (Origene, MD, USA) was used for the luciferase assays. Site directed mutagenesis was used to introduce the P91S, A543G, and R585L variants into the wild type *GATA6* construct (Quik Change kit, Stratagene, CA, USA). Primer sequences utilized for mutagenesis are included in Table 1. Following introduction of the variant, the entire coding region was sequenced to ensure there were no additional variants. For the A177T variant, a 130 base pair oligonucleotide was synthesized by Genscript USA, which was then ligated into the double-restricted wild-type *GATA6* construct.

The functional effect of the variant was analyzed by performing luciferase assays using three luciferase reporter constructs, *NPPA*-promoter-luc, nautriuretuc peptide B

(NPPB)-promoter-luc, and the alpha subunit of myosin heavy chain (αMHC)-promoter-luc. *NPPA*, *NPPB* and *αMHC* are downstream target genes for GATA transcription factors and harbor GATA dependent promoters (Charron, Paradis, Bronchain, Nemer, & Nemer, 1999). The *NPPA*, *NPPB* and *αMHC* promoter sequences (0.9 kb, 1.3 kb and 0.7kb respectively) were amplified by a polymerase chain reaction (PCR) and subcloned into a pGL4.10 plasmid. Chinese hamster ovary (CHO) cells were transfected according to manufacturer specifications using Lipofectamine LTX (ThermoFisher, Waltham, MA, USA) with empty expression vector, wild-type *GATA6* or mutant *GATA6* expression vectors, and either the *NPPA*-promoter-luc, *NPPB*-promoter-luc, or *αMHC*-promoter-luc reporter constructs. 500ng of each construct was transfected into multiple replicates in a 24-well plate. 100ng of the pRL-SV40 vector was also co-transfected in each group of cells as an internal transfection control.

Additionally, the wildtype construct and each variant construct was also co-transfected with either T-box transcription factor 5 (*TBX5),* GATA binding protein 4 *(GATA4),* NK2 homeobox 5 (*NKX2.5) or* Myocyte specific enhancer factor 2C *(MEF2C)* constructs and either the *NPPA*-promoter-luc, *NPPB*-promoter-luc, or *αMHC*-promoter-luc reporter constructs.

Firefly and Renilla luciferase activities were measured 24 hours post-transfection using the dual luciferase reporter assay system (Promega Corp, WI, USA). For statistical analyses, two-way ANOVA was performed followed by multiple comparisons with Bonferroni correction. A p-value of <0.05 was considered to be significant.

Table 1: Primers for Site-directed Mutagenesis

	Primer sequence
R585L forward	TCACGTCCTCCGTGCTACCGGATTCCTGGTGCGCC
R585L reverse	GGCGCACCAGGAATCCGGTAGCACGGAGGACGTGA
P91S forward	CATCCCTTCGGGGCTTCCCACGGACCTTCG
P91S reverse	CGAAGGTCCGTGGGAAGCCCCGAAGGGATG
A543G forward	AGGGGCGGGTGGCCCGGTGATGA
A543G reverse	TCATCACCGGGCCACCCGCCCCT

Base substitutions are indicated in red.

Confocal microscopy and Western blot analysis was also performed on wildtype CHO cells and CHO cells that had been transfected with each mutant construct.

VI. Functional Assay of HHATL

RNA was extracted from each cell line used and then reverse transcribed to cDNA using ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswitch, MA, USA). Inherent expression of Hedgehog acyl transferase (*HHAT)*, Hedgehog acyl transferase-like (*HHATL)*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a positive control was then determined using reverse transcription polymerase chain reaction (RT-PCR). Different sets of primers (Table 2) were designed for each cell line using Primer3Plus (Antergasser and Nijveen, 2007). For each primer pair the PCR settings were as follows, 95°C for 5 mins, then 95°C for 30 seconds, 60°C for 1 minute, and 68°C for 1 minute (40 cycles), and finally 68°C for 5 minutes.

Table 2. Primers for RT-PCR	
HEK293_HHAT_F	GCTGCCCCGATGGGAACTGG
HEK293_HHAT_R	AGGAGCTGAGACCGGAACTGGG
HEK293_HHATL_F	CGGGGCCTTGGCTGTGATGG
HEK293_HHATL_F	CTGCCTGGGCTCGGATGTGC
HEK293_GAPDH_F	TGGTCGTATTGGGCGCCTGG
HEK293_GAPDH_R	GCCAGGGGTGCTAAGCAGTTGG
COS7_HHAT_F	TGGACTCACTCCACCCGCCC
COS7_HHAT_R	GAGGGTCACCCAAGGCCAGC
COS7_HHATL_F	GCCTTCCGGGAGTCTGTGCG
COS7_HHAT_R	GTGGGCGCAGCTCTCCAGTG
COS7_GAPDH_F	TGGTCGTATTGGGCGCCTGG
COS7_GAPDH_R	GCCAGGGGTGCTAAGCAGTTGG
CHO_HHAT_F	TGCTGCCCCGATGGGAAATGG
CHO_HHAT_R	GCAGCCTCAGGGTGGAGAGC
CHO_HHATL_F	AAGTTTGCCAGCCGCCTCCC
CHO_HHATL_R	AGCCGGGCCTCGAATTGTGC
CHO_GAPDH_F	CGCCCCTTCTGCTGATGCCC
CHO_GAPDH_R	GTGGGCCCTCAGATGCCTGC

The Sonic hedgehog (*Shh*) gene was received from plasmID at Harvard Medical School in a pENTR223.1 plamid. The *HHAT* and *HHATL*, and mutant *HHATL* constructs were synthesized by Gen 9 Bio (Cambridge, MA) in the pG9M plasmid. The original plamids were subjected to PCR to isolate the each desired gene. The *Shh*, *HHATL* and *HHATL* mutant constructs were subcloned into the pKH3 plasmid while the *HHAT* construct was subcloned into the pCDNA3 plasmid. The pKH3 plasmid was digested at the Xba and EcoRI sites and the pCDNA3 plasmid was digested at the XbaI and BamHI sites. Then, each gene was inserted into their respective plasmid by a Gibson reaction.

The *HHATL* and *HHATL* mutant constructs came with a FLAG-tag, however *Shh* and *HHAT* did not so the FLAG-tag was added manually using PCR primers (Table 3).

Table 3. Primers for Flag Tagging	
Shh_Flag_F	cttctgcaggtcgactgccaccATGCTGCTGCTGGCGAGA
Shh Flag_R	catggcggccatcgattgtcaGCTGGACTTGACCGCCATG
HHAT_Flag_F	caagcttggtaccgagctcgGCCACCATGCTGCCCCGA
HHAT_Flag_R	acactatagaatagggccctCTACTACTTGTCGTCATCGTCTTT GTAGTCG

Human embryonic kidney cells (HEK293), CHO and African green monkey kidney fibroblasts (COS7) cells were cultured according to recommendation. HEK293 ($6.25 \text{ X } 10^5$), COS7 ($2.5\text{X}10^5$), and CHO ($2\text{X}10^5$) cells were seeded onto a 6-well plate (35 mm/well) and transfected with each plasmid with Lipofectamine LTX and Plus reagent (ThermoFisher, Waltham, MA, USA).

These cells were then lysed with lysis buffer containing 25 mM Tris-HCL, 5 mM EDTA, 5mM EGTA, 1% Triton X-100, 150 mM NaCl, 20 mM NaF, 0.2-1 mM Na3VO4, 20 mM glycerol-2-phosphate, and protease inhibiter (Boston BioProducts, Ashland, MA, USA). The protein concentrations were then measured using a DC Protein Assay and iMark Microplate Reader (Bio-Rad, Hercules, CA, USA).

10 µg of each sample was then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-15% Mini-PROTEAN TGX polyacrylamide gels followed by a transfer to a polyvinylidene difluoride membrane using the Trans-Blot

Turbo Transfer System (Bio-Rad, Hercules, CA, USA). They were then blocked using 5% skim milk in NaCl/P_i (PBS) containing 0.1% Tween.

To determine the amount of modified 19-kDa *Shh*-Np the blots were probed with 1:1000 H-160 and 5E1 antibodies along with anti-Flag, anti-*GAPDH* protein, and anti-enhanced green fluorescent protein (EGFP) as internal controls. The blots were incubated overnight in an antibody/PBS/0.1%tween/5% dried milk solution. The blots were then washed in PBS/0.1% Tween 2 x 15 mins each. Next the blots were incubated with secondary antibodies at 1:10000 for 2 hours at room temperature. The blots were then washed again in PBS/0.1% tween 3 x 15 mins each and once with PBS for 5 minutes. The blots were analyzed using a Chemiluminescent Nucleic Acid Detection Module Kit (ThermoFisher Scientific, Waltham, MA) and ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA).

The antibodies used were monoclonal anti-*Shh* N-terminal fragment (5E1, 1:1000; Developmental Studies Hybridoma Bank at the University of Iowa); rabbit anti-Shh N-terminus (H-160, 1:1000; Santa Cruz Biotechnology Inc, Santa Cruz, CA); monoclonal anti-EGFP (JL-8, 1:1000; Fisher Scientific, Pittsburgh, PA); polyclonal anti-*GAPDH* protein conjugated with horseradish peroxidase (HRP) (FL-335,1:1000; Santa Cruz Biotechnology Inc, Santa Cruz, CA); HRP conjugated goat anti-mouse IgG (sc-2031,1:10000, Santa Cruz Biotechnology Inc, Santa Cruz, CA); HRP-conjugated goat anti-rabbit IgG (ab6721-1, 1:10000; ABCAM, Cambridge, MA)

RESULTS

The clinical data from family AF-772 (Figure 3) are summarized in Table 4. Five members of the family were affected and the trait appeared to be inherited in an autosomal dominant fashion. Individual 7, the proband, was diagnosed with an ostium primum ASD. Her children, 71 and 72 were diagnosed with AF at a young age, 14 and 16 respectively. The probands sister, 2, had a single documented paroxysm of AF. The probands nephew, 22, had a VSD during childhood.

Table 4. Clinical Characteristics of AF-772 Family Members

ID	Sex	Age	Phenotype	PR	QRS	QT	LVEF (%)	LA size (mm)	LVID (mm)	RV base (mm)	R585L variant
1	F	67	-	122	68	436	66	31	36	38	N
2	F	66	AF	162	72	421	72	37	42	29	Y
21	M	33	-	122	92	416	-	-	-	-	Y
22	M	31	VSD	144	116	400	60	37	48	27	Y
3	F	65	Abnormal ECG*	130	84	426	77	33	40	27	Y
4	M	64	-	-	-	-	-	-	-		N
43	F	27	-	130	82	444	73	31	46	33	N
5	F	62	-	148	86	447	66	34	45	32	N
6	M	60	-	154	88	422	78	37	44	28	N
61	M	28	-	144	104	434	64	37	50	37	N
62	F	25	-	154	76	394	68	30	43	31	N
7	F	58	ASD	146	90	437	55	53	43	-	Y
71	M	28	-	-	-	-	-	-	-	-	-
72	M	25	AF	152	104	405	55	40	54	-	Y
73	M	22	AF	148	94	401	49	33	53	-	Y

AF, atrial fibrillation; HR, heart rate; LVEF, left ventricular ejection fraction; LA, left atrium; RV, right ventricular

*Abnormal ECG with P wave inversion in lateral leads and right ventricular conduction delay

From an analysis by our lab an average of 18,825 variants were found in individuals 22, 72, and 73 via exome sequencing (Table 5).

Table 5: Number of Candidate Variants Identified by Exome Sequencing

Individual	**22**	**72**	**73**
Total no. of variants	18,529	18,019	19,928
No. of variants following filtration based on population databases	803	771	592
No. of missense variants	548	521	385
No. of nonsense variants	15	7	8
No. of splice site variants	0	1	7

Filtering these variants to remove common, synonymous variants reported in population databases (dbSNP, 1000 Genomes, Exome Variant Server, and the EXac database) and variants that did not segregate with disease yielded a short list of 13 novel or very rare missense variants that individuals 22, 72, and 73 have in common (Table 6). Further prioritization was then based on gene expression profiles, interspecies conservation, predicted effect on protein function, and potential involvement with cardiac related functional pathways.

Of these potentially interesting variants *GATA6,* Titin *(TTN), HHATL*, and Coiled-coil domain containing 69 (*CCDC69)* had high cardiac expression (i.e. protein present in cardiomyocytes). Ten variants were located on residues conserved throughout evolution (a Phylop score of greater than 0).

Table 6: List of Potentially Causative Genetic Variants in Family AF-772

Gene	Amino Acid change	Description	Conservation Score (Phylop)	Polyphen prediction	Cardiac Expression*	rsID	ExAC Frequency
GATA6	R585L	GATA binding protein 6	4.94	Deleterious	High	rs201707559	2.44E-05
ZNF704	Y130*	Zinc finger protein 704	3.09	N/A	Intermediate	rs200928219	
TTN	R12327C	Titin	2.14	Deleterious	High	rs72646859	0.000662
MUC4	G1212A	Mucin 4, cell surface associated	1.998	N/A	Low		
NHLRC3	R165*	NHL repeat containing 3	1.184	N/A	N/A	rs143048352	4.07E-05
STOX2	R480S	Storkhead box 2	0.882	Deleterious	Intermediate	rs199772096	0.002008
SBF1	R888H	SET binding factor 1	0.714	Deleterious	Low	rs201519518	0.000231
MUC4	A1209V	Mucin 4, cell surface associated	0.572	Deleterious	Low	rs200800552	
HHATL	K484N	Hedgehog acyltransferase-like	0.555	Deleterious	High		
CCDC69	R7K	Coiled-coil domain containing 69	0.295	Neutral	High		3.25E-05
TOPAZ1	N430T		-0.272	Neutral	N/A	rs200454057	0.000269
SLC38A10	L707P	Solute carrier family 38, member 10	-0.96	N/A	Intermediate		
MUC4	S2661N	Mucin 4, cell surface associated	-2.704	N/A	Low	rs201201006	0.000353

*Expression levels from GNF Expression Atlas 2 data from U133A and GNF1H chips in UCSC browser

A more comprehensive segregation analysis was performed for five of the 13 variants with high cardiac expression, a positive Phylop score, and had plausible links to cardiac disease after examination of the literature. Segregation analysis was performed by Sanger sequencing each region of interest in all 16 family members to determine whether or not they carried the variant. These results are included in Table 7.

Table 7: Segregation Analysis of Five Candidate Variants in Family AF-772

	Cardiac Phenotype	GATA6 R585L	TTN R12327C	NHLRC3 R165*	HHATL K484N	CCDC69 R7K
2	AF	+	+	+	+	+
3		+	+	+	-	+
7	ASD	+	+	+	+	+
9		+	+	+	+	-
21		+	+	+	-	+
22	VSD	+	+	+	+	+
72	AF	+	+	+	+	+
73	AF	+	+	+	+	+
1		-	+	-	-	-
4		-	-	+	-	+
5		-	-	-	+	+
6		-	+	+	-	-
8		-	+	-	-	+
43		-	-	+	-	-
61		-	+	-	-	-
62		-	+	-	-	-

Based on the segregation analysis and prioritization the R585L *GATA6* was found to be the most compelling variant followed by *HHATL* as a close second (Tables 6 and 7 and Figures 4 and 5).

There were no significant differences between electrocardiogram (ECG) parameters (PR, QRS and QT intervals) between mutation carriers and patients without the mutation (*GATA6*-R585L genotype positive vs genotype negative; PR; 143±13 vs 142±13 ms, p=0.85; QRS; 93±14 vs 84±12 ms, p=0.24; QT; 451±14 vs 430±19 ms, p=0.15).

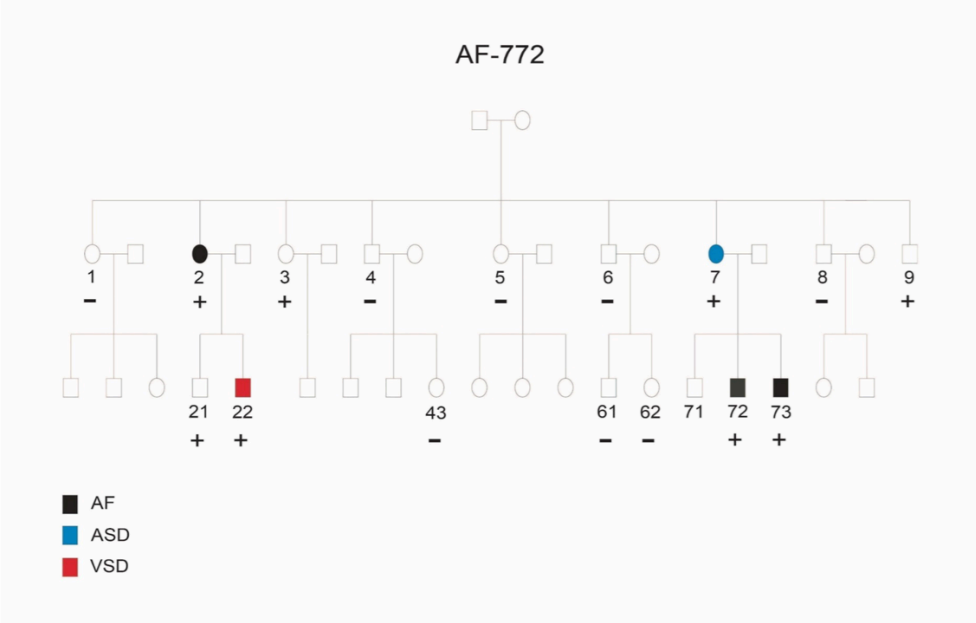

Figure 4. *GATA6* R585L variant segregation pattern in AF-772

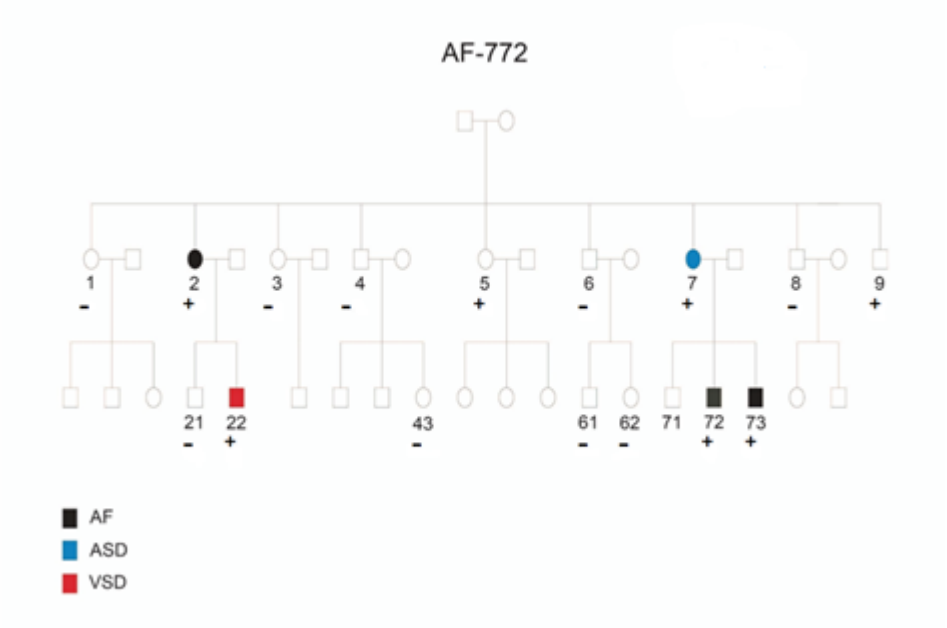

Figure 5. *HHATL* K484N variant segregation pattern in AF-772

MGH AF Study

Given our finding in AF-772 we screened a cohort of 546 patients with early onset AF. All patients in this cohort developed AF before the age of 66 and had structurally normal hearts. Table 8 includes a summary of the clinical characteristics of this cohort. We found three additional variants in the *GATA6* gene in three separate individuals: A543G, P91S, and A177T.

Table 8. Clinical Characteristics of Individuals with Early-Onset AF from the MGH AF Study

Study	MGH AF Study
Number	546
Female	113 (20.7)
Mean age, years	54.0 ± 10.5
Mean age, onset AF	47.1 ± 10.9
Family history of AF	246 (45.1)
Hypertension	167 (30.6)
Heart failure	18 (3.3)
Diabetes	27 (4.9)

Values are n (%) or mean ± SD.

Table 9. Clinical Characteristics of Variant Carriers from the MGH AF Study

Amino Acid	Study Number	Sex	Age of onset (years)	AF Pattern	EF (%)	LA Size (mm)	QRS axis	IVCD
P91S	438	F	54	Permanent	63	NA	47	No
A177	441	M	47	Paroxysmal	72	43	51	LBBB
A543G	184	M	61	Paroxysmal	67	44	42	No

Table 10. Location, frequency and conservation of GATA variant

Exon	Nucleotide	Amino Acid	rs ID	Phylop	Polyphen	GERP
2	C271T	P91S	rs200620299	0.38	0.007	1.59
2	G529A	A177T	-	1.34	0.991	2.36
7	C1628G	A543G	-	-0.13	0.010	-0.30
7	G1754A	R585L	rs201707559	2.02	0.992	3.12

None of the patients had a family history of AF. Abbreviations: EF, ejection fraction; LA, left atrial; NA, Not available.

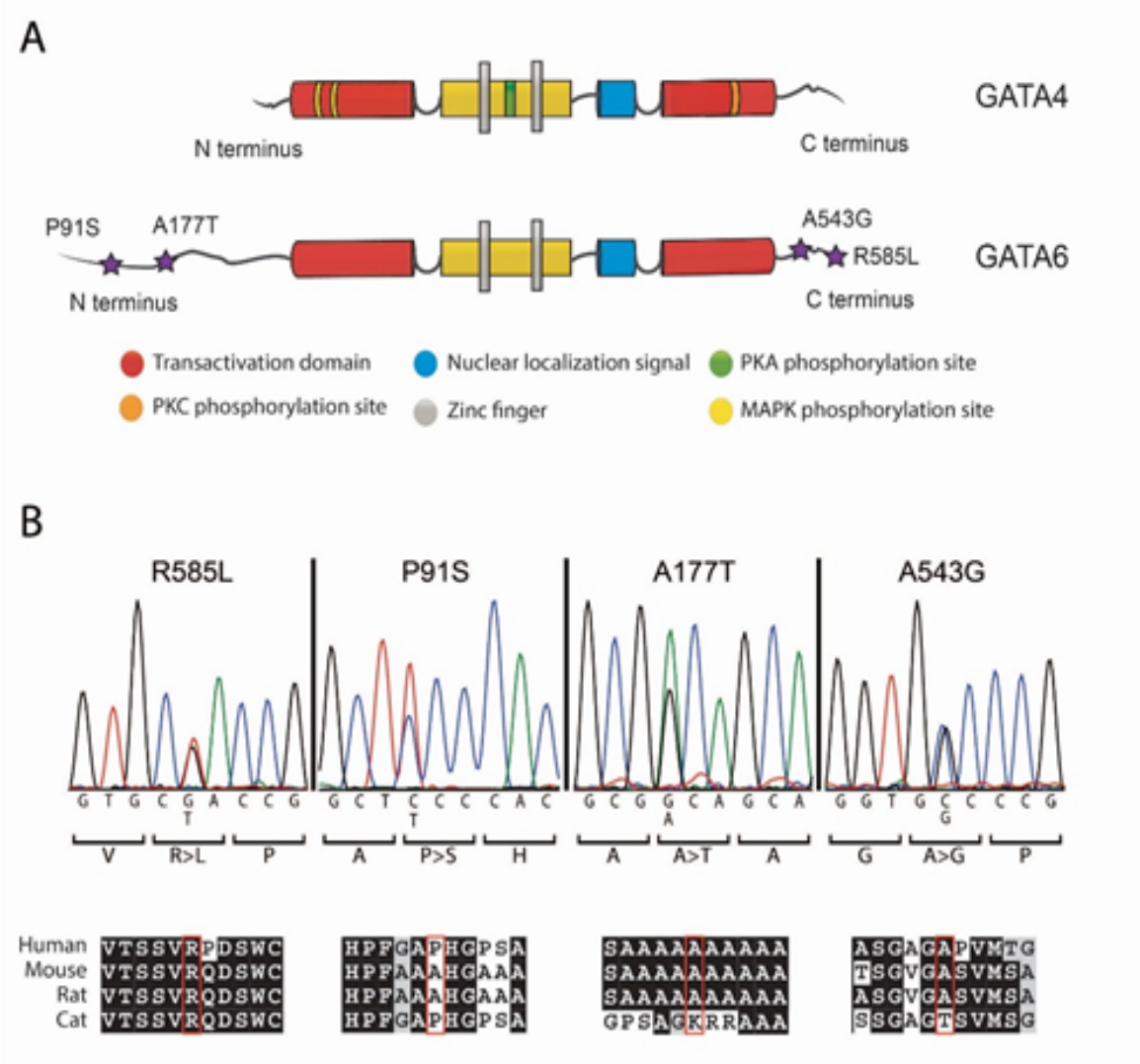

Figure 6. Summary of *GATA6* variants found both in AF-772 and MGH cohort.

In order to determine these variants effects on protein function we performed a functional analysis of the variants found in *GATA6*. To do this we evaluated the ability of exogenously expressed wild-type and mutant *GATA6* to activate known GATA family-targeted promoters upstream of a luciferase reporter. We found that certain variants were concomitant with an upregulation in luciferase activity in NPPA (R585L: 4.1 fold, $p<0.0001$, P91S: 2.5 fold, $p=0.0002$, A117T:1.7 fold, $p=0.03$) and *αMHC* (R585L: 1.7 fold, $p=0.03$, P91S; 1.7 fold, $p=0.03$) promoters (Figure 7). None of the variants showed any change in NPPB promoter activity. Also, the A543G mutant displayed no net effect in any of the constructs as compared to control.

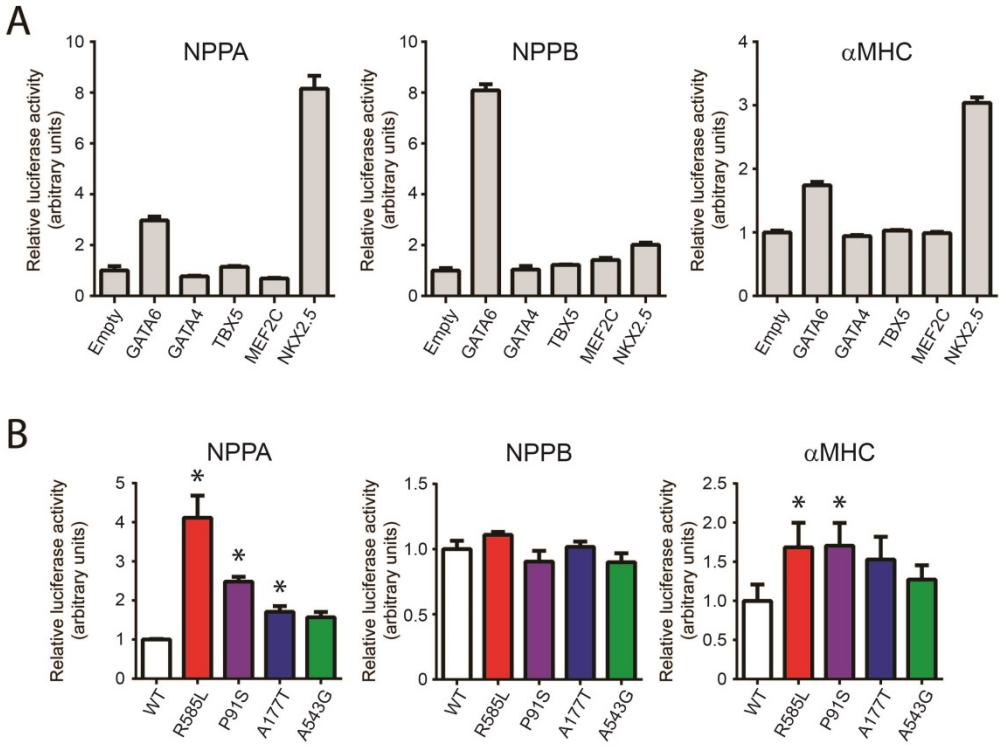

Figure 7. A: Relative luciferase activity recorded from CHO cells following cotransfection of reporter constructs with *GATA6, GATA4, TBX5, MEF2c, NKX2.5*, or empty vector. B: Luciferase assay results for *GATA6* wildtype and variants in *NPPA*, *NPPB* and *αMHC* promoters.

In order to determine the mechanism of these functional effects we examined two common events that can cause changes in transcriptional activity: altered subcellular localization of the protein and differences in overall levels of the protein's expression. However no difference was observed in subcellular localization or levels of protein expression as compared to the wildtype based on confocal microscopy and Western blot respectively (Figure 8).

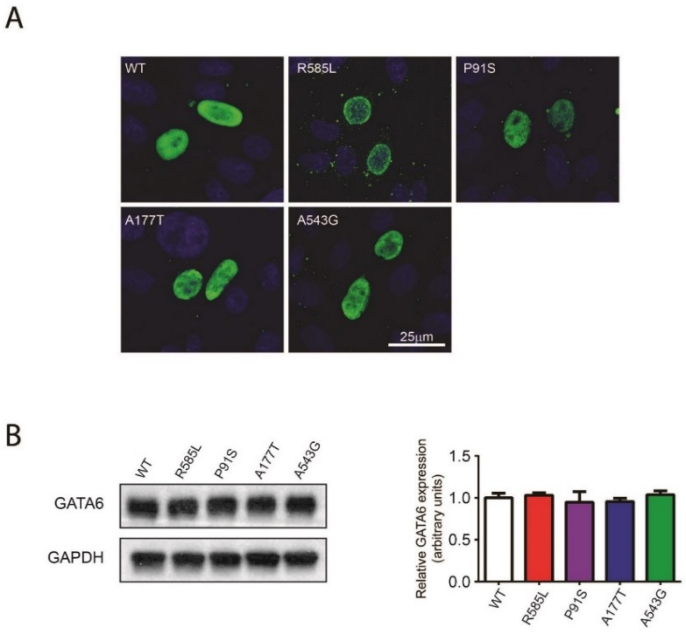

Figure 8. A: Subcellular localization of *GATA6* wildtype compared to *GATA6* variants. B: Levels of protein expression in *GATA6* wildtype versus *GATA6* variants.

Wildtype *GATA6* is found in the nucleus, the same results were found with overexpression of each of the variants in CHO cells. Similarly, Western blots of cellular lysate probed for *GATA6* show that all of the constructs are expressing *GATA6* at the same relative level.

Because *GATA6* is a transcription factor and transcription factors often do not work alone and come into contact with other transcription factors we sought to examine the effect of co-transfecting our *GATA6* mutant constructs and other common transcription factors *GATA6* is known to come in contact with. These transcription factors include: *TBX5* (Maitra et al., 2009), *GATA4* (Charron, Paradis, Bronchain, Nemer, & Nemer, 1999), *NKX2.5* (Molkentin et al., 2000), and *MEF2C* (Morin, Charron, Robitaille, & Nemer, 2000).

A difference in NPPA promoter activity was observed when *GATA6* R585L and P91S variants were co-transfected with *NKX2.5* or *TBX5* and, more importantly, when all variants were co-transfected with *GATA4* or *MEF2C*. A difference in *NPPB* promoter activity was observed only when R585L was co-transfected with either *TBX5* or *MEF2C*. Finally a difference in αMHC promoter activity was observed when each *GATA6* variant was co-transfected with *TBX5, GATA4, or MEF2C* (Figure 9.) When expressed independently of *GATA6, TBX5, GATA4 and MEF2C* had negligible effects of expression of the reporter constructs (Figure 7A).

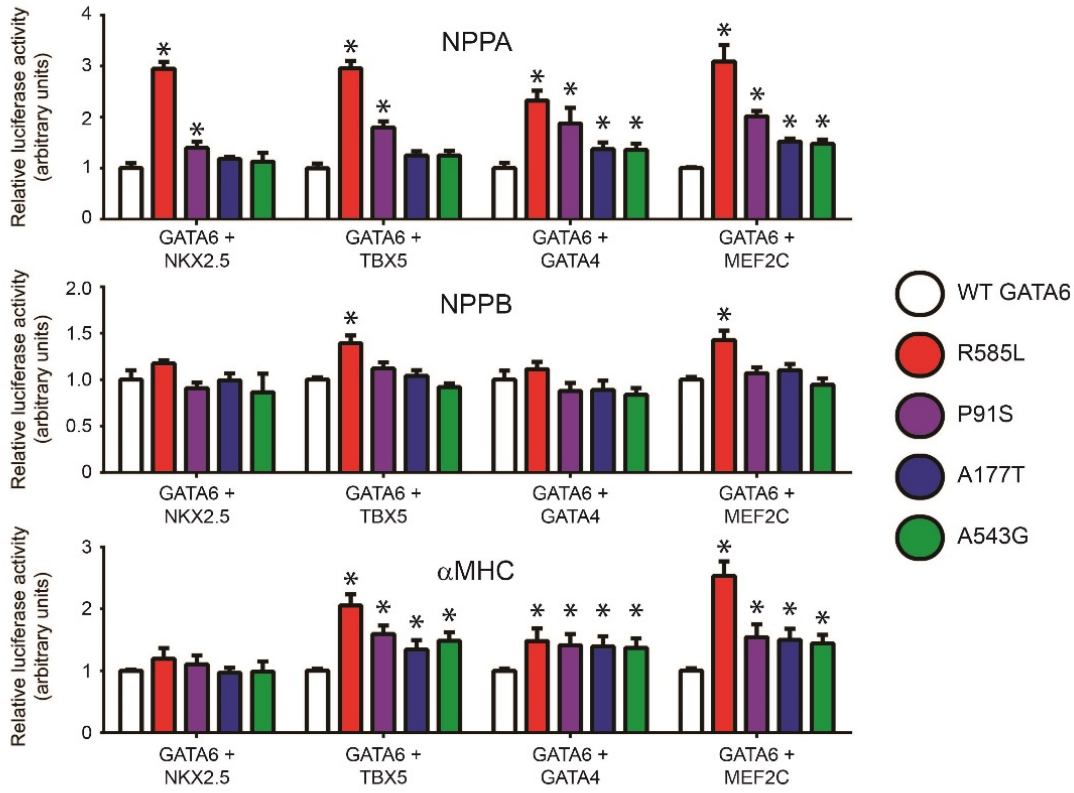

Figure 9. Luciferase assays of *GATA6* wildtype and variants co-transfected with either *NKX2.5*, *TBX5*, *GATA4*, or *MEF2C* with three different promotor constructs (*NPPA*, *NPPB*, and *αMHC*).

A functional analysis of the *HHATL* variant was then performed based on previously described methods (Abe, Kita, & Niikura, 2008).

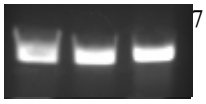

Figure 10. Inherent expression of *HHAT, HHATL*, and *GAPDH* in HEK293, COS7, and CHO cells.

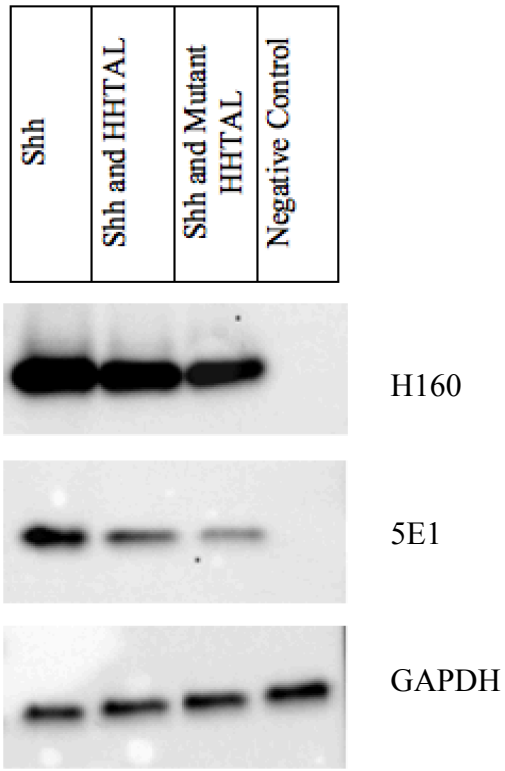

Figure 11. Western blot of *HHATL* wildtype activity compared to *HHATL* variant activity in COS7 cells.

DISCUSSION

By exome sequencing AF-772 and subsequently filtering and prioritizing the variants we were able to narrow down the disease causing mutation to two variants in two different genes, *GATA6* and *HHATL*. We also found three additional variants in the *GATA6* gene in a cohort of patients from MGH with early onset AF. Age is significant in this regard because mutations in younger patients with AF have greater genetic dependence with respect to their phenotypic expression (A Palatinus & Das, 2015).

GATA6 is a known transcription factor and that contains a highly conserved DNA binding domain which consists of two zinc finger motifs that bind to the nucleotide sequence element (A/T)GATA(A/G) (Molkentin, 2000). Transcription factors help to initiate gene transcription and play a vital role in terms of gene expression. *HHATL* (hedgehog acyl transferase-like) inhibits *HHAT* (hedgehog acyl transferase), an important protein involved in the post-translational modification of Sonic Hedgehog (*Shh*), an important signaling protein involved in embryonic patterning and organogenesis. *HHAT* palmitoylates Sonic Hedgehog on its N-terminal Cys residue. This lipid modification affects Shh's diffusibility, movement and concentration gradient and is therefore essential to Shh's signaling capabilities during embryonic development (Abe, Kita, & Niikura, 2008). Therefore, it is plausible that the mutant *HHATL* loses its ability to inhibit *HHAT*, which could in turn, affect *Shh* signaling during development and result in cardiac defects.

However, based on our functional assay results the *HHATL* mutant (did not cause any change in its functional activity. This would imply that the *HHATL* K484N is not responsible for AF-772 disease. However, further conformation of these results is needed.

On the other hand, all four *GATA6* variants we found exhibit gain-of-function modulation in luciferase assays (a measure of transcriptional activity) with up-regulation of *NPPA* and *αMHC* promoter activity. This could potentially be attributed to some sort of altered synergistic interaction between the variants and other transcription factors that *GATA6* comes in contact with (Figure 9). For example, when all *GATA6* variants were co-transfected with *TBX5, GATA4, or MEF2C* a difference in *αMHC* promoter activity was observed versus without co-transfection we only saw a difference *αMHC* promoter activity with R585L and P91S. This indicates that the presence of certain transcription factors can unmask a differential in some *GATA6* variants' activity that wouldn't otherwise be detectable.

Interestingly, these *GATA6* variants were found in the N-terminus and C-terminus of the protein. This is noteworthy because these regions are thought to be important regulators of the protein's function. If the variants were found in the zinc finger or transactivation domains they would likely cause detrimental effects that may not be compatible with life. Many previous studies further corroborate our findings that *GATA6* is the causative mutation in AF-772. GATA transcription factors have been found to play an important role in cardiac development and morphogenesis; mainly by activating the expression of terminal differentiation markers (Laverriere et al 1994). Additionally, *GATA6* was found to have a key role in regulation differentiation of heart precursor cells;

when *GATA6* levels were artificially elevated terminal differentiation in the heart is blocked (Gove, 1997). This is significant because it could lead to abnormal differentiation of cardiomyocytes which can contribute to triggering arrythmias (Zhang, Hartzell, Narlow, & Dudley, 2002).

It has also been found that protein expression of *GATA6* was dramatically increased in the adult mouse heart in response to pressure overload. The same study also examined the effect of a deletion of *GATA6* in the adult heart and found a significant reduction in hypertrophic growth response, indicating a role in pathological cardiac hypertrophy (Berlo et al., 2010). Another more recent study demonstrated similar results when they found that Sofroaphane decreased cardiac hypertrophy and also decreased levels of *GATA6* protein (Kee, Kim, Kim, & Jeong, 2015). Another study showed *GATA6* gene expression in stem cell derived SA and AV nodal cardiomyocytes (Hashem & Claycomb, 2013). Importantly, these studies shows that, in addition to having an important role during development, *GATA6* also regulates gene expression in the adult heart and it's possible that the *GATA6* gene plays some sort of role in maintaining the electrical conduction system in the adult heart.

GATA6 variants have previously been implicated in cardiac abnormalities and congenital defects including atrioventricular septal defects and Tetrology of Fallot, and patent ductus arteriosus (Maitra, Koenig, Srivastava, & Garg, 2010) (Wang et al., 2012) (Kodo et al., 2009). Importantly, in addition to studies linking *GATA6* mutations with structural cardiac defects, candidate-gene studies have identified four variants in the *GATA6* gene in patients with AF. One study examined a cohort of 110 Chinese Han

patients with AF and found two novel variants (Q206P and Y265X), as compared to the control population (Yang et al., 2012). Another study the same group found an additional variants (Y235S) in a separate cohort. Similarly, Lin et al found another mutation in *GATA6* (G469V) in patients with lone AF. The variants G469V and Y235S have been functionally validated, and in contrast to our findings, they resulted in a loss of *GATA6* function.

Limitations

Individuals 21, 3, and 9 carried the *GATA6* variant but did not display any overt structural cardiac abnormalities or arrhythmias. Individual 3 did however demonstrate a very abnormal P wave axis with P wave inversion in the lateral leads (I and AVL) and evidence of right ventricular conduction delay. Individuals 9 and 21 had normal baseline ECGs. These findings may suggest that the *GATA6*-R585L variant is incompletely penetrant. However we cannot make this conclusion without further clinical evaluation. Perhaps these individuals may develop AF later on in life. Without more extensive clinical evaluation we were unable to exclude subtle septal defects and asymptomatic episodes of AF in these individuals.

The complex phenotype of the family is also difficult to assess. Since congenital heart disease often leads to AF, could the mutation that was found in this family be causing congenital heart disease which in turn makes them more susceptible to AF? It's possible that individuals 2, 72, and 73 had an undetected heart defect contributing to their AF. However, the more likely explanation is that different mutations can manifest themselves different in individuals depending on their genetic make-up. Previous studies

have also found that a specific mutation in a transcription factor can result in multiple different phenotypes (Seidman & Seidman, 2002).

Additionally, the R585L identified in Family AF-772 and the P91S and A543G variants found in individuals with early-onset AF, were identified in rare individuals in the 1000 Genomes Project and the ExAC database, but not in the Exome Variant Server. This highlights the limitations of using SNP repository data as a "control" population in the absence of clinical data. It is possible that the individuals with these variants in the databases could have AF, and ASD, or VSD. However, the transmission of R585L in the family as well as the clear gain-of-function effect strongly support the role of this variant in the pathogenesis of the phenotype.

Further studies

As stated previously, it would be interesting to re-evaluate the family members more comprehensively and see if any of them (particularly individuals 21, 3, and 9) have developed AF or any other heart problems.

Analyzing the phenotype of a cardiac specific mouse knock-out, knock-down, and overexpression of *GATA6* would give us a better understanding of *GATA6*'s role in the heart. Additionally, electrophysiological studies on transgenic mice with the specific mutations we found in AF-772 and the MGH cohort would be a beneficial functional model of the variants.

Another potential avenue could be to create knock-down and overexpression lines of GATA6 in cardiomyocytes to see if cardiomyocyte differentiation patterns are impacted or if there is any cellular phenotypic difference, such as prolonged APD.

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CURRICULUM VITAE

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EDUCATION

Boston University School of Medicine—Boston, MA

Masters in Medical Sciences, 2016 (anticipated)

Bachelor of Science in Human Physiology, May 2014

PROFESSIONAL EXPERIENCE

Cardiovascular Research Center at Massachusetts General Hospital, Charlestown, MA

Research Assistant 2011-2013 (Summers) and Present

Dr. Patrick Ellinor, Primary Investigator

- Screened the genes GATA6, ADAM15 for mutations in a cohort of families with atrial fibrillation using high-resolution melting technology. Found two novel mutations in GATA6, manuscript in preparation
- Worked with post-docs, Dr. Jordan Leyton-Mange and Dr. Nathan Tucker.
    - Assisted with optical mapping using ArcLight protein, to categorize cardiac action potentials using fluorescence microscopy. We used distinct populations of differentiating cardiomyocytes to report pharmacological effects of selected drugs.
    - Used the CRISPER-Cas9 nuclease system as a new tool for genome editing in zebrafish, which can produce mutations with higher fidelity and less off target effects.

Medical Shadowing

Massachusetts General Hospital, Boston, MA

January 2012

- Observed rounds and diagnosis discussions on Oncology, Thoracic surgery, general surgery services, and both surgical and medical intensive care units.
- Shadowed nurse practitioners and physician assistants in all of the above settings as well as in an academic primary care medical practice.

- Observed and interacted with post-op thoracic surgery and primary care medical outpatients.

VOLUNTEER WORK

bWell Center, Boston Medical Center, Boston, MA

Volunteer, January 2015-Present
- Patient education and activities coordinator
- Ran the center during my shift by engaging children in various activities that promote wellness and encourage a healthy lifestyle.
- Provide information and access to various health resources for patients and their families

Allston/Brighten Congregational Church, Brighton, MA

Server, January 2015-Present
- Create food baskets to be distributed (two Saturdays a month)
- Work with a volunteer team to prepare and serve dinner at the parish's Community Supper once a week.

Massachusetts Eye and Ear Infirmary, Boston, MA

Volunteer, January 2014-April 2014
- Worked in the surgery center helping the surgical nurses with pre-op and post-op patients
- Assisted nurses on the inpatient floor with daily patient-care duties
- Patient transport

SKILLS

- **Laboratory**: High Resolution DNA melting, subcloning, optical mapping, PCR, DNA sequencing, western blotting, cell culture, immunohistochemisty

- **Computer:** Microsoft office, Zotero, RefSeq

- **Languages**: Conversant in Spanish

AWARDS

2014-Sargent College of Heath and Rehabilitation Dean's List