Epistatic interaction of *PDE4DIP* and *DES* mutations in familial atrial fibrillation with slow conduction

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Abstract

Background: The genetic causes of atrial fibrillation (AF) with slow conduction are unknown. Methods: Eight kindreds with familial AF and slow conduction, including a family affected by early onset AF, heart block and incompletely penetrant non-ischemic cardiomyopathy (NICM) underwent whole exome sequencing. Results: A known pathogenic mutation in the desmin (*DES*) gene resulting in S13F substitution at a PKC phosphorylation site was identified in all four members of the kindred with early-onset AF and heart block, while only two developed NICM. Higher penetrance of the mutation for AF and heart block prompted the screening for *DES* modifier(s). A second deleterious mutation in the phosphodiesterase 4D interacting-protein (*PDE4DIP*) gene resulting in A123T substitution segregated with early onset AF, heart block and the DES mutation. Three additional novel deleterious *PDE4DIP* mutations were identified in four other unrelated kindreds. Characterization of PDE4DIP^{A123T} in vitro suggested impaired compartmentalization of PKA and PDE4D characterized by reduced colocalization with PDE4D, increased cAMP activation leading to higher PKA phosphorylation of the β2-adrenergic-receptor, and decreased PKA phosphorylation of Desmin in response to isoproterenol stimulation compared to wildtype PDE4DIP. Conclusion: Our findings identify an epistatic interaction between *DES* and *PDE4DIP* variants, increasing the penetrance for conduction disease and arrhythmia.

1. Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia in the general population, with an estimated lifetime incidence of 7% to 26%. It is associated with major cardiovascular complications, including stroke, tachyarrhythmia and heart failure (Go et al., 2013). While AF typically leads to a rapid ventricular response in the majority of patients, a subset of AF is associated with a slow heart rate (Amat-y-Leon et al., 1974; R. K. Kumar, Saxena, & Talwar, 1991; Yamashita, Murakawa, Ajiki, & Omata, 1997).

AF has a major genetic component with a mode of inheritance of a complex trait and in rare cases as a single gene disorder. More than 44 disease-causing genes with rare damaging mutations have been identified in monogenic forms of familial AF. In addition, over 95 genetic loci have been identified by genome wide association studies of AF (Alzahrani et al., 2018). The pathophysiology of the distinct clinical entity characterized by AF and slowing of conduction along the electrical conduction system is poorly understood. Conduction slowing leading to early onset heart block may occur at the time of birth with an incidence of 1 in 15,000- 25,000 live births (Costedoat-Chalumeau, Georgin-Lavialle, Amoura, & Piette, 2005) and is often associated with maternal anti-Ro/SSA and/or anti-La/SSB autoantibodies, and is less commonly due to congenital syphilis, rheumatic fever or diphtheria infection (Michaelsson, Riesenfeld, & Jonzon, 1997).

Progressive conduction system disease, which may lead to heart block is heritable and has been associated with mutations in the genes encoding cardiac ion channels (Baruteau, Probst, & Abriel, 2015). Other forms of conduction system disease develop later in childhood or early adult life and are associated with dilated cardiomyopathy (Moak et al., 2001; Udink ten Cate et al., 2001) or AF but its etiology remains poorly understood and are likely caused by different genetic mutations. However, the genetic causes of AF with slow ventricular response are vastly unknown.

We describe a family, which was referred to us for genetic testing due to the co-existence of early onset AF and heart block requiring pacemaker implantation in the fourth decade of life. Additionally, several family members developed non-ischemic cardiomyopathy. The genetic causes of these traits were investigated using whole exome sequencing (WES). In addition, seven independent kindreds with AF and conduction disease not requiring pharmacologic rate control underwent WES.

2. Materials and Methods

Study Subjects

Patients with early onset familial AF, including a multiplex Lebanese family with early onset familial AF, cardiomyopathy, and heart block requiring pacemaker placement were referred to the Yale Cardiovascular Genetics clinic for genetic screening. All other kindreds had isolated AF with no structural heart disease. The protocol was approved by the institutional review board at Yale University School of Medicine. Informed consent to participate in the study and to undergo genetic sequencing was obtained from all patients. Detailed clinical information, including laboratory data and clinical imaging were collected. Genomic DNA was extracted from peripheral blood leukocytes and sent for exome sequencing. Family history was obtained from the index cases, and pedigrees were constructed based on self-reported phenotypes.

Whole Exome Sequencing and Targeted Sequence Capture

Genomic DNA was captured on exomes at the W.M. Keck Facility at Yale University using Roche NimbleGen 2.1M Human Exome Array, as described earlier (Keramati et al., 2014). In brief, DNA libraries were prepared and sequenced on the Illumina Genome Analyzer, followed by image analysis and base calling. Sequences were aligned against human reference genome (UCSC Genome Browser hg19) and processed using MAQ program SAMtools. SAMtools was also used for the single-nucleotide variant detection and filtering against the reference genome as described earlier. Filters were applied against published databases.

Exome analysis

Segregation analysis was performed on all three phenotypes of interest in the Lebanese family, which may exhibit epistatic interaction in development of cardiomyopathy, heart block and AF. A computer script was designed for variants annotation based on the novelty, conservation, tissue expression and their effect on protein function. They were considered non-conservative if the substituted amino acid was conserved in all species. Filtering criteria included frequency (<1/1000), and splice site location (-2 to +2 intron exon boundary). Synonymous, intronic variants, and variants with low quality were filtered out. Variants that were predicted to be benign by two in silico tools (SIFT, and Polyphen2) as well as by other by other laboratories were filtered out early in the analysis. CADD score was additionally used to predict pathogenicity and variants with a score of less than 10 were excluded. Regions prone to sequencing errors were screened then all the related duplications and deletions were filtered out. Also, all variants in known AF-associated genes were screened for allele frequencies <1% in the Gnomad database. Lastly, intolerance score and phyloP 46-way score were used as a measure of conservation. The GTEx portal was used to filter out genes without cardiac tissue expression.

Familial AF kindreds and mutation burden analysis

The candidate gene lists for segregating AF and heart block in the Lebanese family were further narrowed down by screening an additional exome dataset of 7 kindreds with AF and conduction disease. The same

filtering criteria for the identified genetic variants was used. Mutation burden analysis was then carried out in cases versus 158 ethnically matched controls via Fisher Exact test.

In vitro FRET imaging of cAMP signaling

The PDE4DIP NM_001002811 transcript was cloned into an mCherry-tagged plasmid and a point mutation, resulting in an alanine to threonine substitution at amino acid 123, was induced using site directed mutagenesis and verified by sequencing. Human embryonic kidney (HEK) cell line 293T was transfected with the PDE4DIP mutant and control plasmids using the Lipofectamine transfection kit. Transfection efficiency was verified by percentage m-cherry expression on fluorescent microscopy (>50%). The cells were then co-transfected with the FRET sensor plasmids using the Lipofectamine kit and incubated on 35mm microwell dishes with 14mm glass coverslip for 3 days to achieve 60-70% confluency.

Intracellular cAMP imaging was done using FRET sensors and confocal microscopy at baseline, and at 4 minutes after stimulation with 1uM isoproterenol (cAMP activator). Further imaging was done 4 minutes after stimulation with 25 uM of Forskolin (strong cAMP activator) under live confocal microscopy. A fourth generation Epac-based FRET sensor (mTurquoise2Δ-Epac (CD, ΔDEP, Q270E)-tdcp173Venus (Epac-SH187)) was used as previously described (Klarenbeek, Goedhart, van Batenburg, Groenewald, & Jalink, 2015). This sensor consists of a full-length cAMP-binding Rap-1 activating protein Epac, which is sandwiched between the donor and acceptor fluorescent proteins with larger conformational change, and FRET change compared to sensors with partial Epac. Fourth generation of cAMP sensors have superior photo-stability, dynamic range and signal-to-noise ratios. The design is based on mTurquoise2 as a bleaching-resistant donor, and a tandem of two cp173Venus fluorophores as acceptors.

FRET was quantified using 3 cube ratiometric imaging with analysis in MATLAB using previously developed custom software (A. Kumar et al., 2016). Images were acquired on an inverted Nikon Eclipse Ti widefield microscope equipped with a cooled charged-coupled device Cool SNAP HQ2 camera using a 20x 0.75 NA objective at 37°C. Three sequential images were acquired with the following filter combinations: donor (mTurquoise2) channel with 460/20 (excitation filter-ex), T455lp (dichroic mirror-di) and 500/22 (emission filter-em); FRET channel with 460/20 (ex), T455lp (di) and 435/30 (em); and acceptor (Venus) channel with 492/18 (ex), T515lp (di) and 535/30 (em) filter combinations. For analysis, donor leakage was determined from 293T cells transfected with mTurquoise2 and acceptor cross excitation was obtained from Vinculin-Venus transfected cells. All three FRET images (mTurquoise2, Venus, FRET) were background subtracted and filtered by three-point smoothening. FRET maps and pixel-wise FRET index was calculated as:

FRET index = [FRET channel – x(Donor channel) – y(Acceptor channel)]/[Acceptor channel]

Where x is the leakage co-efficient and y is the cross-excitation fraction. Masks for each cell were generated by thresholding mCherry positive cells using the mTurquoise images. Mean FRET index per cell was calculated for each field under each treatment condition.

Immunohistochemistry

The C2C12 cells were reverse transfected with lipofectamine:DNA complexes prepared according to the manufacturer's protocol (ThermoFisher, lipofectamine 3000). The C2C12 cells were trypsinized, resuspended in media (DMEM+10%FBS, without antibiotics). The single cell suspension was added to the lipofectamine mix at a concentration of 10^5 cells/well of 6-well dish. The cells were plated on a 6-well plate and allowed to adhere overnight. Next day, the media was changed to DMEM+10%FBS, with antibiotics. The cells were treated with either DMSO or isoproterenol at the concentration of 1μM for 8 minutes. The cells were washed with 1XPBS, and fixed with 4% formaldehyde overnight at 4°C. The cells were permeabilized with 1XPBS-0.1%Trition, 3X, 5' each, followed by blocking in 1XPBS-0.1%Trition+10%FBS for 1 hour and overnight incubation in primary antibody at 3 μgm/ml (Pde4D, catalog# PA521590 , Lot #VH3049391A), followed by washes in 1XPBS-0.1%Trition, 3X, 5 minutes each, and overnight incubation in secondary antibody with conjugated Alexa-488. The cells were washed again in 1XPBS-0.1%Trition, 3X, 5 m minute each and imaged with SP8 confocal microscope. The immunostaining for p-Desmin for done as described above except

for p-Desmin primary antibody used at a concentration of 200 µgm /ml (p-Desmin Ser60, ThermoFisher, Catalog# PA5-38837).

Western Blotting

The C2C12 cells were transfected with mutant and control PDE4DIP plasmids (PDE4DIP-MUT and PDE4DIP-WT respectively) using lipofectamine and cultured in a 100 mm plates. At day 3, cells reached 70% confluency and plasmid expression was verified by percentage mCherry fluorescence (>50%) using confocal microscopy. Cells were stimulated with isoproterenol (1 uM) and harvested in protease and phosphatase inhibitor buffers at 8 minutes. Cells were quickly collected, placed in liquid nitrogen and subsequently prepared for western blot analysis. The western blot was carried out using standard procedures. Western blot analysis was performed to quantify phosphorylation changes in the beta-2-adrenergic receptor using the antibodies for p-beta adrenergic receptor Ser355, 356 (Catalog # PA538403, ThermoFisher), p-beta adrenergic receptor Ser346 (Catalog#Ab19281, Abcam), total beta-adrenergic receptor (Ab182136, Abcam) at a concentration of 1 µgm /ml.

3. Results

3.1 Pathogenic *Desmin (DES)* mutation and its association with cardiac traits

The index case of the Lebanese family was a 48-year-old man (III-2, figure 1) who presented with syncope and was diagnosed with heart block. Interestingly, his underlying cardiac rhythm at the time was AF. No reversible factors were identified for the AF or heart block. He underwent a permanent pacemaker placement and has been doing well to this date at age 54 without cardiomyopathy. Examination of the remaining family revealed very similar presentations in his 52-year-old brother (III-1, figure 1) and maternal uncle (II-2) who developed heart block with AF at 40 years of age, necessitating a permanent pacemaker with a major difference that they both developed non-ischemic cardiomyopathy (NICM) also in their mid-40 years of age. The mother (II-1) also had similar presentations with early onset heart block and AF but no cardiomyopathy. A 60-year-old maternal aunt (II-6) had AF at 57 years of age but no cardiomyopathy or conduction disease. In sum, there were 4 living family members with heart block and early onset AF, with only 2 of them having NICM, one of whom died shortly after the ascertainment.

Of note, there were 2 other maternal uncles who had died prior to the ascertainment; one of them had known heart block (II-3) and had died in a car accident at 40 years of age, whereas the other (II-4) had an implantable cardiac defibrillator device for his heart failure and died at age 62 from end stage heart failure. While they carried the diagnosis of unspecified arrhythmia, a history AF could not be verified. Most recently, one young family member (III-5) developed NICM with left bundle branch block, and an old family member (patient II-2) was diagnosed with AF at 57 years of age but without conduction disease or NICM.

All four subjects with early onset AF harbored a known pathogenic mutation in the *Desmin* (*DES*) gene (NC_000002.11:g.220283222C>T) that resulted in a serine to phenylalanine substitution at codon 13 (p.S13F). A healthy 65-year-old maternal uncle (II-5) who underwent exome sequencing was negative for the *DES* mutation (figure 1).

Interestingly, the *DES* mutation leading to p.S13F substitution had been previously reported in 8 other families affecting a total of 45 patients (supplementary table 1) (Bergman et al., 2007; Pica, Kathirvel, Pramono, Lai, & Yee, 2008; van Tintelen et al., 2009). On average, 38% of patients harboring the p.S13F substitution developed heart block requiring pacemaker implantation at an average age of 41 years, and 38% of patients developed cardiomyopathy (mostly dilated and arrhythmogenic right ventricular cardiomyopathy) at an average age of 44.6 years, but only 11% of patients had AF in their disease course (supplementary table 1). Furthermore, only 31% had mild proximal and distal skeletal myopathies (supplementary table 1). A more comprehensive review of all known mutations throughout the *DES* gene indicated that on average 49% develop cardiomyopathy, 36% develop conduction disease requiring pacemaker placement, and a minority (<10%) develop AF (van Spaendonck-Zwarts et al., 2011). While the incidence of cardiomyopathy in the Lebanese pedigree (50%) was similar to that reported in the literature, the incidence of heart block (67%)

requiring pacemaker and AF (83%) were much greater.

3.2 Epistatic interaction between *DES* and *PDE4DIP* mutations

The remaining mutations were filtered as described in the methods to remove common variants and genes not expressed in the heart and performed segregation analyses of the remaining genes. This revealed a mutation in the *PDE4DIP* gene that segregated in all 4 affected family members with early onset heart block and AF. The *PDE4DIP* mutation (NM_001002811:c.367G>A) results in a non-conservative alanine to threonine substitution at amino acid 123 (p.A123T). The presence of *PDE4DIP* mutation in all family members with very early onset AF and heart block suggested an epistatic interaction with the *DES* gene, resulting in increased penetrance of these two traits. Another member of the family (II-6), who was found to be a carrier of the *DES* mutation had only developed late onset AF at age 60 but has neither NICM nor conduction disease (figure 1). One young unaffected 32-year-old member of the kindred who has been considered as too young to develop disease (III-5) was found to be a carrier of both mutations *DES* and *PDE4DIP* mutations and is being closely followed by a local cardiologist.

PDE4DIP is an anchoring protein that interacts with both phosphodiesterase 4D (PDE4D) and cAMP dependent protein kinase A (PKA) (Dodge et al., 2001) and several other proteins to form a multiprotein complex that plays an important role in targeting signaling processes to subcellular locations. PDE4D hydrolyzes cAMP and regulates its levels within cardiac myocytes where it also complexes with proteins mediating sympathetic signals to heart, including β-adrenergic receptors (Mongillo et al., 2004; Perry et al., 2002; Xiang et al., 2005).

A novel deleterious heterozygous variant of the *ENG* gene (NC_000009.11:g.130578055G>A) causing a p.A628V substitution also co-segregated with cardiomyopathy. The *ENG* gene encodes a membrane glycoprotein primarily expressed in the vascular endothelium and myocardium and has been previously identified as a modifier gene for hypertrophic cardiomyopathy (HCM) in patients with pathogenic MYH7 mutations, resulting in more profound myocardial fibrosis (Frustaci, Lanfranchi, Bellin, & Chimenti, 2012).

3.3 *PDE4DIP* Mutations Among Patients with early onset AF and mutation burden analysis

We subsequently screened 7 kindreds with AF and slow ventricular response suggestive of conduction disease using WES. All variants with allele frequencies greater than 1/1,000 or considered as benign by PolyPhen and Sift, including a number of variants in *PDE4DIP* were filtered. There were 3 independent novel deleterious nonconservative mutations in *PDE4DIP* gene that segregated with the disease in 4 kindreds (table 1, figure 2 and 3). A mutation burden analysis was then carried out between WES variants of all cases compared to 158 age and ethnically matched controls via Fisher Exact test. Statistical analysis showed significantly higher burden of mutations in *PDE4DIP* gene in cases vs. controls (adjusted p value <0.05). No other variants AF or heart block genes were identified in the 7 kindreds.

3.4 Functional characterization of the *PDE4DIP* mutation

We first examined whether the A123T substitution alters the interaction between PDE4D and PDE4DIP, using immunofluorescence staining and specific antibody against PDE4D. Wildtype (WT) and mutant *PDE4DIP* were expressed in C2C12 cells and the expression was verified by positive mCherry auto-fluorescent tag. Careful examination showed that upon isoproterenol stimulation, PDE4D and wildtype PDE4DIP show increased colocalization but the co-localization of PDE4D and PDE4DIP^{pA123T} was dramatically reduced (Figure 4).

To assess how the altered interaction between PDE4DIP^{A123T} and PDE4D affects cAMP levels, cells were transfected with a plasmid either containing the *PDE4DIP*^{A123T} or wild type *PDE4DIP* . Since cAMP activation occurs at compartmental level within the cells, the effect of PDE4DIP^{A123T} on intracellular cAMP signaling was explored using a fluorescence resonance energy transfer (FRET)-based reporter. Isoproterenol was used to stimulate the endogenous beta-2 adrenergic receptor (β2AR). The FRET sensor imaging showed increased cAMP levels in mutant compared to wildtype transfected cells in response to isoproterenol stimulation (Figure 5).

Examination of the PKA and the and G protein-coupled receptor serine/threonine kinases (GRKs) phosphorylation sites on the β2AR revealed an increase in PKA mediated phosphorylation but no significant difference in β2AR phosphorylation at the GRK residues in cells expressing PDE4DIP^{A123T} compared to wild type PDE4DIP (figure 6). The examination of the PKA phosphorylation of Desmin by immunofluorescent microscopy revealed decreased phosphorylation at its serine 31 site and diminished colocalization of Desmin with PDE4DIP (Figure 7). The reduced phospho-Desmin in the mutants suggests that PDE4DIP^{A123T} mutation causes loss of compartmentalization of both PDE4D and PKA, resulting in increased PKA phosphorylation of β2AR but reduced phosphorylation of Desmin. These findings are highly relevant since inactivation of sarcoplasmic PDE4D and altered Desmin phosphorylation have both been linked to cardiomyopathy and arrhythmias (Beca et al., 2011; Lehnart et al., 2005; Rainer et al., 2018).

4. Discussion

In this manuscript we describe the identification of *PDE4DIP* as the genetic modifier of *DES* . Desmin is a type III intermediate filament protein that plays a pivotal role in the electromechanical functioning of cardiomyocytes (Mado et al., 2019). It connects the Z discs, which are at the center of the contractile unit, to the plasma membrane, nucleus and mitochondria and plays a pivotal role in the contractile function of cardiomyocytes. Mutations in *DES* are associated with autosomal dominant dilated cardiomyopathy with incomplete penetrance. Over 40 pathogenic *DES* mutations have been causally linked to myofibrillar myopathies involving skeletal myopathies, non-ischemic cardiomyopathies and cardiac conduction disease (van Tintelen et al., 2009). The p.S13F substitution occurs within a highly conserved motif in the non-helical head domain of the protein that is shared by type III and IV intermediate filaments (Bar, Strelkov, Sjoberg, Aebi, & Herrmann, 2004; Pica et al., 2008). This substitution eliminates serine at a known phosphorylation site for protein kinase C, thus interfering with the protein organization and likely leading to deleterious Desmin aggregation and disruption of the cytoskeletal network (Kitamura et al., 1989). Although Desmin is widely known as a skeletal muscle protein it is highly expressed within heart and within the conduction system of the heart and the pulmonary vein myocardial sleeves that are foci of origin of AF (Kugler et al., 2018). Accordingly, the mutations have pleotropic effects with cardiomyopathy being the most prominent trait, followed by cardiac conduction disease and much less commonly AF (van Spaendonck-Zwarts et al., 2011). The genetic modifiers of *DES* have not been identified. Interestingly, six members of the Lebanese kindred who were carriers of the pathological Desmin p.S13F substitution had slightly higher incidence of NICM compared to the literature (50% vs 38% in published data), but considerably higher incidence of early onset AF (83% vs 11% in published data) and heart block requiring a pacemaker (67% vs 38% in published data) (supplementary table 1) (Bergman et al., 2007; Pica et al., 2008; van Tintelen et al., 2009). The high incidence of AF and heart block suggested epistatic interactions between the *DES*mutation and other genetic mutations and provided an exceptional opportunity to search for genetic modifiers.

Our study identified the *PDE4DIP*^{pA123T} mutation as a genetic modifier of *DES* that increases the penetrance of heart block and early onset AF in *DES* mutation carriers. Also known as myomegalin, *PDE4DIP* is highly expressed in cardiac atrial and ventricular tissue (supplementary figure 1). It is a large gene located on the long arm of chromosome 1 and contains 64 exons coding for a 2,362 amino acid protein that was first isolated in 2001 in a study of cardiac genes (Soejima et al., 2001) with several splicing variants . The protein is composed of alpha-helical and coiled-coil structures and is also heavily expressed in skeletal muscles (supplementary figure 1). Within myocytes, PDE4DIP is predominantly localized in the Golgi compartment, in proximity to the cytoskeletal apparatus and in the nucleus (Bouguenina et al., 2017; Soejima et al., 2001; Wang, Zhang, & Qi, 2014; Wu et al., 2016). It is an A-kinase-anchoring protein that is involved in the assembly of the cAMP dependent protein kinase A (PKA)/phosphodiesterase 4D (PDE4D) cAMP signaling module in a multiprotein complex (Dodge et al., 2001). Co-compartmentalization of both PKA and PDE4D is critical for sustained specificity of adrenergic signaling to subcellular locations, contractility of cardiomyocytes and timely termination of the second messenger response (Fink et al., 2001).

PDE4D hydrolyzes cAMP and regulates its levels within cardiac myocytes where it also complexes with proteins mediating sympathetic signals to the heart, including β-adrenergic receptors (Mongillo et al., 2004;

Perry et al., 2002; Xiang et al., 2005) . The activity of PDE4D has been also localized to the transverse (T) tubule/sarcoplasmic reticulum (SR) junctional space thus mediating cAMP/calcium homeostasis that is involved in excitation-contraction coupling (Mika, Richter, & Conti, 2015; Mongillo et al., 2004; Zaccolo & Pozzan, 2002). Of note, loss of function of PDE4D is associated with cardiomyopathy and AF (Jorgensen, Yasmeen, Iversen, & Kruuse, 2015).

We further established an association between multiple rare non-conservative *PDE4DIP* mutations with large effects in four out of seven unrelated families referred for early onset familial AF and slow conduction without structural heart disease. The functional significance of these mutations was established by the combined statistical power, high conservation of the mutated bases, and in vitro functional studies.

The *in vitro* characterization of PDE4DIP^{pA123T}suggested a gain of function mutation leading to increased PKA activity. This effect is likely due to the disruption of the spatial-temporal activity of PDE4D, which is normally anchored to PDE4DIP and showed reduced colocalization with PDE4DIP^{pA123T}. Consequently, there was increased cAMP activation by PDE4DIP^{pA123T} vs. wildtype PDE4DIP in response to isoproterenol as assayed by FRET imaging. Under normal circumstances, the rise of cAMP in response to isoproterenol triggers PDE4D activation as well as desensitization of the G-protein coupled β2AR receptor via phosphorylation by PKA and G protein-coupled receptor serine/threonine kinases (GRKs) at distinct phosphorylation sites (Xin, Tran, Richter, Clark, & Rich, 2008). PKA mediated phosphorylation of the β2AR at Ser345/346 by PKA causes a switch from stimulatory Gs to inhibitory Gi protein (Zamah, Delahunty, Luttrell, & Lefkowitz, 2002). On the other hand, prolonged β2AR stimulation results in its GRK mediated phosphorylation at Ser 355/356, its binding to β-arrestin, termination of G protein-mediated signaling, and facilitated receptor endocytosis resulting in receptor desensitization (Nobles et al., 2011; Shenoy & Lefkowitz, 2005). In addition, PDE4D binds to β-arrestin and is subsequently recruited to the plasma membrane to regulate PKA mediated phosphorylation of β2AR.(Houslay & Baillie, 2005; Perry et al., 2002; Willoughby et al., 2007). Interestingly, there was more pronounced phosphorylation of the β2AR at the PKA residue (Ser 346) in the mutant versus control cells while the phosphorylation at the GRK residues (Ser355-356) was unchanged. The former may underlie the mechanism of slow heart rate in patients with AF.

Like all A-kinase-anchoring proteins, PDE4DIP binds to the regulatory subunit of PKA and anchors it to the N-terminal region of cMyBPC, whereby it mediates cMyBPC phosphorylation and plays an important role in regulation of cardiac contractility. The PDE4DIP colocalization with cMyBPC and cTNI has been shown to increase upon β adrenergic activation (Uys et al., 2011). Desmin is also known to be a phosphorylation target for cAMP-dependent kinases (Gard & Lazarides, 1982; O'Connor, Gard, & Lazarides, 1981). Specifically, PKA phosphorylation of Desmin regulates its function and assembly (Inagaki et al., 1988; Tao & Ip, 1991). Interestingly, our studies in C2C12 cells showed that the β adrenergic activation reduces colocalization between mutant PDE4DIP and phospho-Desmin compared to wildtype PDE4DIP. This suggests that PDE4DIP directs PKA to Desmin, but PDE4DIP^{pA123T} induced loss of compartmentalization of both PDE4D and PKA leads to PKA phosphorylation of β2AR but reduced phosphorylation of the Desmin, which has also lost a PKC phosphorylation site by the p.S13F mutation. Phosphorylation changes in Desmin are shown to alter its assembly and trigger accumulation of toxic preamyloid oligomers in acquired heart failure (Bouvet et al., 2016). Thus, the perturbed compartmentalization of PDE4DIP^{pA123T} and further reduction in Desmin phosphorylation is a mechanistic link to the modifier effect of the PDE4DIP variant on the mutant Desmin, leading to increased penetrance of heart block and AF in Desmin p.S13F carriers.

It is noteworthy that a common nonsynonymous variant (rs1778155) of PDE4DIP gene, resulting in arginine for histidine substitution at codon 1761 has been associated with ischemic stroke in NHLBI Exome Sequence Project (odds ratio: 2.15; p-value: 2.63×10^{-8}) (Auer et al., 2015). This disorder is believed to be associated with AF. Thus, our findings may provide a molecular link between ischemic stroke and slow AF, which is often subclinical due to the absence of tachycardia and palpitations.

Our success in identifying the disease gene and its modifier was largely due to our systematic study of outlier subjects with well-characterized disease and the segregation analysis of the rare variants. The investigated kindreds in our study all had early onset AF with slow conduction and demonstrated an autosomal dom-

inant pattern of inheritance. This approach is particularly superior to case–control association studies for identification of disease-causing rare variants of large genes with high mutation burden such as *PDE4DIP* . It is, however, noteworthy that not all identified segregating *PDE4DIP*mutations may have sufficient power to be independently disease causing; this is particularly true for variants with higher allele frequencies.

One limitation of our study is that it does not examine the effect of the PDE4DIP mutation on K channel and ryanodine receptor RyR2. PDE4D has been shown to regulate the slow delayed rectifier K current (Terrenoire, Houslay, Baillie, & Kass, 2009) and RyR2 (Lehnart et al., 2005; Mika, Richter, Westenbroek, Catterall, & Conti, 2014). Its inhibition leads to increased PKA activation of the slow delayed rectifier K current (Terrenoire et al., 2009), a mechanism that may underlie the AF (Sampson et al., 2008). It remains to be determined if PDE4DIP mutation generate a functional substrate for AF in the atrium by altering K current. In addition, a Mendelian randomization study examining link between rs1778155 variant, AF, and stroke could support the potential link between PDE4DIP common variant and the risk for atrial fibrillation and should be a topic for future investigations.

5. Conclusion and Future Prospects

Desmin mutations have pleotropic effects and often exhibit incomplete penetrance, but modifier genes that explain this behavior had not been identified. In this study, we show epistatic interaction between*DES* and *PDE4DIP* mutations. In addition, we find increased mutation burden of the *PDE4DIP* gene in kindreds with slow AF. The described mutations in this gene impaired the spatially regulated cAMP dependent PKA activation and altered phosphorylation of several proteins that play important roles in cardiac contractile function and conduction. In summary, our study has implicated PDE4DIP in maintenance of cardiac function and as a potential target for development of novel drugs for treatment of arrhythmias and cardiomyopathies.

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Tables:

Table 1. Rare damaging *PDE4DIP* mutations identified in 4 independent kindreds with familial slow AF. Het: heterozygous, Hom: homozygous, PolyPhen 2: Polymorphism Phenotyping v2 software.

PDE4DIP Gene Mutation	Amino Acid substitution	Het/Hom	Prediction by PolyPhen 2	1,000 Genomes	EX
NC_000001.10:g.144931342C>T	A123T	Het	Damaging	Novel	2.4
NC_000001.10:g.144877255T>C	I1478V	Het	Damaging	Novel	4.7
NC_000001.10:g.144866687C>T	R1852Q	Het	Damaging	Novel	5.2
NC_000001.10:g.144881520G>A	R1226C	Het	Damaging	Novel	5.7

Figures legends:

Figure 1. Lebanese family with atrial fibrillation, heart block and non-ischemic cardiomyopathy. All patients underwent exome sequencing and the mutations were confirmed by sanger sequencing, III-2 is the index case. E1: evaluation for S13F in *DES* and E2: evaluation for A123T in *PDE4DIP* .

Figure 2. Pedigrees with early onset atrial fibrillation and slow ventricular response and their corresponding PDE4DIP mutations. The index cases are indicated by arrows. Individuals with slow AF are indicated by black symbols; unaffected individuals are shown as unfilled symbols. Circles represent females; squares represent males. Symbols with a slash through them indicate deceased subjects.

Figure 3. Segments of the protein sequences flanking the substituted amino acids in the four kindreds with slow AF are shown from diverse vertebrate species. As demonstrated, all substitutions in the familial AF kindreds occurred at evolutionarily conserved sites.

Figure 4. Colocalization of PDE4DIP with PDE4D in C2C12 cells expressing wildtype and mutant (p.A123T) PDE4DIP before and 8 minutes after isoproterenol exposure. The arrows show colocalization of PDE4DIP with PDE4D in wildtype and the absent thereof in the mutant C2C12 cells.

Figure 5. Fluorescence resonance energy transfer (FRET) imaging of Isoproterenol (ISO 1uM) and Forskolin (FOR 25uM) activation of cAMP. Images were obtained under live confocal microscopy at baseline and at 4 minutes after stimulation with 1uM ISO. Further imaging was done 4 minutes after stimulation with 25 uM of Forskolin. The upper and lower limits of the FRET ranges are shown on the right in the colored keys. The quantification of 18-20 fields of two independent experiments per groups are shown in bar graph. FRET imaging shows a significant increase of cAMP activity (decreased FRET) in cells transduced by the mutant (p.A123T) compared to wild type PDE4DIP after stimulation with Isoproterenol (ISO 1uM).

Figure 6. Western blot analysis of β2AR phosphorylation. A, shows increased β2AR phosphorylation at the PKA but not GRK sites in PDE4DIP mutant cells compared to wildtype 8 minutes after stimulation with isoproterenol (1uM). B, the ratio of β2AR phosphorylation at PKA site (S346) to the total β2AR shows increased baseline phosphorylation in the wild type but much higher levels of phosphorylation in the mutant following isoproterenol stimulation. C, the ratio of β2AR phosphorylation at GRK sites (S355, S356) to the total β2AR shows increased baseline phosphorylation in the wild type but no significant increase in the

phosphorylation levels in the mutant after isoproterenol stimulation compared to wild type or control. The "*" indicates significant changes when comparing unstimulated to post-isoproterenol signal.

Figure 7. Reduction of total phospho-Desmin in C2C12 cells transduced with the mutant (p.A123T) versus wildtype PDE4DIP. Red: PDE4DIP mCherry, green: anti phospho-Desmin antibody staining.

Figure 8. Graphical abstract.

Data Availability Statement:

Data available on request due to privacy/ethical restrictions.

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.