A Zebrafish Forward Genetic Screen Identifies an Indispensable Threonine Residue in the Kinase Domain of PRKD2

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Summary statement: We identified, through a zebrafish forward screen, an evolutionarily conserved residue in the catalytic domain of protein kinase D2 and its homologues.

Abstract: Protein Kinase D2 belongs to a family of evolutionarily conserved enzymes regulating several biological processes. In a forward genetic screen for zebrafish cardiovascular mutants, we identified a mutation in the prkd2 gene. Homozygous mutant embryos develop as wild-type up to 36 hours post-fertilization and initiate blood flow, but fail to maintain it, resulting in a complete outflow tract stenosis. We identified a mutation in the prkd2 gene that results in a T757A substitution at a conserved residue in the kinase domain activation loop (T714A in human PRKD2) that disrupts catalytic activity and drives this phenotype. Homozygous mutants survive without circulation for several days, allowing us to study the extreme phenotype of no intracardiac flow, in the background of a functional heart. We show dysregulation of atrioventricular and outflow tract markers in the mutants and higher sensitivity to the Calcineurin inhibitor, Cyclosporin A. Finally we identify TBX5 as a potential regulator of PRKD2. Our results implicate PRKD2 catalytic activity in outflow tract development in zebrafish.
Introduction

Some form of Congenital Heart Disease (CHD) complicates up to 20 out of 1,000 live births, with septal and valve defects being the most common form of CHD (Hoffman and Kaplan, 2002), providing a strong rational for studies that unravel fundamental mechanisms of cardiac morphogenesis and valve formation. To this end, various animal models that recapitulate human heart diseases associated with valve defects have been generated. Many studies have focused on atrioventricular (AV) endocardial cushion development, a milestone in cardiac valve formation. While this developmental stage has been studied extensively in mouse and chicken models, several aspects of heart formation are evolutionarily conserved among different classes of vertebrates including in zebrafish (Beis et al., 2015), a model that is highly genetically homologous to human and is uniquely suited for this type of analysis; zebrafish are amenable to non-invasive imaging techniques as well as forward and reverse genetic manipulations. While proper cardiac valve development and morphogenesis depends on the shear-stress imposed on endocardial cells at the valve forming regions as a result of intracardiac flow dynamics (Vermot et al., 2009; Kalogirou et al., 2014), the absence of circulation does not present an obstacle for these studies in zebrafish, since embryo development is maintained via oxygen delivery through passive diffusion (reviewed in (Bournele and Beis, 2016; Giardoglou and Beis, 2019)). Not surprisingly, mutants identified in zebrafish with intracardiac flow defects, also exhibit valve morphogenesis defects with the most representative being the silent heart (sih) mutant. sih carry a mutation in the cardiac troponin T gene (Tnnt2) causing a total absence of cardiac contractility. While the embryos survive for several days and appear morphologically wild-type, both the AV as well as outflow tract valves remain severely hypoplastic (Sehnert et al., 2002; Bartman et al., 2004).

The zebrafish heart has two chambers, a single atrium and a single ventricle. Blood flow enters the atrium through the sinus venosus and passes through the atrioventricular (AV) canal to the ventricle and then exits through an outflow tract consisting of the bulbus arteriosus and the ventral aorta. The AV valve and outflow tract valve prevent blood regurgitation with the AV region expressing differentiation markers at ~37 hours post fertilization (hpf) (Walsh and Stainier, 2001). The first step in valve formation is the generation of endocardial cushions (EC), transient structures that result from expansion of the extracellular matrix (ECM) between the endocardium and myocardium at the AV canal (Beis et al., 2005). Valve endocardial cells migrate into this region and undergo endothelial-to-mesenchymal transition (EMT), becoming valve interstitial cells (VICs).
Studies to date implicate Nfatc1 signaling (Sugi et al., 2004; Timmerman et al., 2004; Gunawan et al., 2020), Notch1 and bone morphogenetic protein 2 and 4 (BMP2/4) signaling pathways (de la Pompa et al., 1998; Chang et al., 2004) and Calcineurin/NFAT-dependent suppression of vascular endothelial growth factor (vegf) as mechanisms that regulate AV canal differentiation, EC formation, and/or EMT (de la Pompa et al., 1998; Chang et al., 2004; Gunawan et al., 2020). While there is evidence that protein kinase D (PRKD) family enzymes are expressed in cardiac valves (Oster et al., 2006) and they contribute to the regulation of a histone deacetylase 5 (HDAC5)-dependent pathway that regulates Notch expression (Just et al., 2011), their precise role in the control of AV valve formation remains uncertain.

PRKD consists of a family of evolutionarily conserved signal-activated enzymes (PRKD1, PRKD2, and PRKD3) (Valverde et al., 1994; Hayashi et al., 1999; Sturany et al., 2001) that play critical roles in fundamental biological processes (Manning et al., 2002) and contribute to the pathogenesis of a large number of clinically important diseases, including pancreatitis (Piscuoglio et al., 2016), various cancers (Yuan and Pandol, 2016), human heart failure development and cardiac hypertrophy (Wei et al., 2015). PRKD isoforms share a modular domain structure consisting of a C-terminal kinase domain and an N-terminal regulatory domain consisting of tandem C1A/C1B motifs that anchor full-length PRKD to diacylglycerol-/phorbol ester-containing membranes (Iglesias et al., 1998; Iglesias and Rozengurt, 1999; Rey and Rozengurt, 2001; Chen et al., 2008) and a pleckstrin homology motif that participates in intramolecular autoinhibitory interactions (Iglesias and Rozengurt, 1998; Waldron et al., 1999). PRKD isoform activation is generally attributed to growth factor-dependent mechanisms that promote diacylglycerol accumulation and protein kinase C- (PKC-) dependent trans-phosphorylation of PRKD at serine residues in the activation loop, a highly conserved 20-30 residue flexible segment in kinase domain that sits near the entrance to the active site and functions to structure the enzyme for catalysis (Rozengurt et al., 2005). Activated PRKD1 and PRKD2 then autophosphorylate at a serine residue in a PDZ domain-binding motif/PRKD consensus phosphorylation motif at the extreme C-terminus. PRKD3 lacks this autophosphorylation site.

While cardiac tissues co-express PRKD1, PRKD2, and PRKD3, and previous studies show that PRKD isoforms can be activated in a stimulus-specific manner in cardiomyocytes (Guo et al., 2011), most studies have focused on the cardiac actions of PRKD1, which is downregulated during normal postnatal development, upregulated in various cardiac hypertrophy/failure models, and contributes to adverse cardiac remodeling and has been implicated in syndromic congenital heart defects (Speliotes et al., 2010; Johnson et al., 2015). Shaheen et al. identified
a homozygous truncating mutation in \textit{PRKD1} that leads to the generation of a catalytically inactive protein (that contains the entire N-terminal regulatory domain but only the first 35 residues at the N-terminus of the kinase domain) in patients with truncus arteriosus (Shaheen et al., 2015). Studies from Sifrim et al. (Sifrim et al., 2016) and more recently Alter et al. (Alter et al., 2020) identify heterozygous de novo missense mutations in \textit{PRKD1} in 5 patients with syndromic congenital heart disease. Of note, 3 of these patients had identical inactivating missense Gly592Arg mutations in the PRKD1 kinase domain.

Studies of the role of PRKD2 in cardiac function and angiogenesis are more limited, but there is some evidence that defective PRKD2 signaling contributes to the development of human hypertrophic cardiomyopathy (Tsybouleva et al., 2004) and PRKD2 activation of a HDAC5 signaling pathway controls the expression of genes involved in Notch signaling during valve formation in zebrafish embryos (Just et al., 2011). This study uses a zebrafish forward genetic screen (Beis et al., 2005) to identify a mutant line that carries an A to G mutation resulting in a T757A substitution of Prkd2 (T714A in PRKD2) that disrupts catalytic activity and results in outflow tract stenosis in zebrafish embryos by 72 hpf.

2. Results

2.1 Impaired heart development in \textit{s411} mutants

The zebrafish \textit{s411} mutant was identified during a large-scale zebrafish ENU-mutagenesis forward genetics screen (Beis et al., 2005). \textit{s411} is a recessive mutation that results in embryonic lethality by 120hpf. \textit{s411} mutants initially appear morphologically as wild-type, but they show a reduced circulation and a heart-specific phenotype at 48hpf which becomes progressively more severe (Figure 1A,B). Specifically, \textit{s411} mutants develop outflow tract stenosis by 72hpf that leads to retrograde blood flow from the ventricle to the atrium and disruption of blood circulation to the rest of the embryo body (supplementary movie 1). As a result, a severe pericardial edema starts developing from 72hpf.

2.2 The \textit{s411} mutants carry T757A mutation in the \textit{prkd2} gene

We performed mapping by chromosome walking, based on microsatellite markers and genetic polymorphism maps in order to identify the recessive mutation that results in the \textit{s411} phenotype. Bulked segregant analysis using a panel of markers across the zebrafish genome, allowed us to map the mutation to chromosome 15 (linkage group 15). Polymorphism marker analyses of individual mutants allowed us to genetically narrow the \textit{s411} mutation to a region between \textit{z6895} and \textit{z51478}. Further, fine-mapping placed this mutation at a site between
markers, CU062627 and BX924136 – a region that contains the candidate genes prkd2, gpr184, 5s rRNA, urgcp and dscam-like1 (Figure 1D). To identify the mutated gene, we sequenced s411 mutant and wild-type (wt) embryos for the genes mentioned above and identified a mutation in the coding region of prkd2. Zebrafish prkd2 consists of 18 exons, encoding for a 923aa protein. The s411 mutation causes an adenine to guanine conversion, and the substitution of a conserved Threonine to Alanine at position 757, in the Prkd2 catalytic domain (corresponding to Thr714 in human PRKD2; Figure 1E and 1F). This threonine, located in the kinase domain activation loop, is evolutionarily conserved across PRKD2 homologs from zebrafish to rodent and human. It is also conserved in the catalytic domain of other PRKD homologs (PRKD1 and PRKD3) and other AGC kinases (including PKA, all PKC isoforms, and AKT). These findings suggested that a Thr757Ala substitution in the Prkd2 catalytic domain might contribute to the s411 mutant phenotype.

In order to confirm that the impaired heart development of s411 mutants derives from the T757A mutation of Prkd2, we injected a prkd2-targeting morpholino at the one-cell stage of wild-type embryos to block the prkd2 mRNA splicing. Morpholino was designed to impair the splicing site of exon 11 with the following intron. Accordingly, the injected embryos display similar phenotype to s411 mutants at a rate of 80% (Figure 1C). They exhibited outflow blood stenosis and blood regurgitation without developing any other phenotypic characteristics phenocopying s411 homozygous mutants. When the cDNA of the injected embryos was sequenced, we verified that exon 11 (53 bp) was removed from the final transcript resulting in out of frame protein following exon10 and a premature stop codon. This structure corresponds to a truncated protein that entirely misses the catalytic site of the enzyme (Figure 1G). In addition, a previously identified mutation in Tyrosine 849 shows a similar phenotype (Figure 1G) (Just et al., 2011).

2.3 The T757A substitution disrupts PRKD2 kinase activity

We performed in vitro kinase assays (IVKAs) to examine the functional consequences of the threonine-to-alanine substitution in the activation loop of Prkd2. Figure 2 shows that WT-PRKD2 is recovered from HEK293 cells with a low level of anti-PRKD1-pSer910 immunoreactivity and CREB-Ser133 kinase activity and that WT-PRKD2 autocatalytic and CREB kinase activities increase in response to treatment with PS/PMA (a pharmacologic activator of PRKD enzymes). In contrast, PRKD2 harboring an activation loop T757A substitution is catalytically inactive; it does not autophosphorylate at its C-terminal autophosphorylation site and it shows no CREB kinase activity (no activity toward a heterologous substrate). Of note,
threonine-to-alanine substitution at the cognate site in PRKD1 results in the identical phenotype – it renders the enzyme catalytically inactive.

2.4 s411 mutants exhibit defects in cardiac morphogenesis and ectopic expression of AV markers

To dissect at the cellular level the phenotypic features of the prkd2 hearts, we performed immunohistochemistry analyses. Using rhodamine phalloidin (filamentous actin staining) and Tg(myl7:dsred) we showed that the ventricular myocardium of prkd2s411 appears to be much thinner at 72hpf compared to wild-type siblings (Figure 3A,A’, B,B’). In addition, we observed that there is a single layer of cuboid AV endocardial cells in mutants whereas wild type embryos appear to have two layers of cuboid cells at the AV canal (Figure 3B,B’). At the outflow tract, there is a complete stenosis occurring at 72hpf (3C,C’), which is the earliest defect we can detect (supplementary movie 2,3). This blocks the circulation towards the branchial vasculature at the level of the intersection between the ventricle and the bulbus arteriosus. Specifically, endocardial cells of this area have collapsed disturbing the continuation of the endocardium to endothelial cells (Figure 3D,D’). In addition, the single layer of cuboidal cells of atrioventricular valve cells in prkd2s411 does not express zn5 - a cell-cell adhesion marker that is localized in myocardial cells and differentiated AV canal cells - in contrast to wild-type embryos (Figure 3E,E’). These data confirms that the outflow tract stenosis and blood regurgitation also affects the morphogenesis of the atrioventricular cardiac valves during development, also as a consequence of the intracardiac flow dynamic breakdown.

We also performed in situ hybridization of notch1, bmp4 and klf2a. The expression of these genes is initially throughout the heart and gets restricted to the AV canal cells by 72hpf. These are the first hallmarks of AV differentiation, and a reliable indicator of proper valve development (Stainier et al., 2002; Beis et al., 2005). All the three markers (notch1b, klf2a, and bmp4) remain ectopically expressed throughout the heart of s411 mutants (Figure 4A’-D’, compare with A-D). In addition, s411 mutants carrying the Tg(TP1:mCherry) (a transgenic reporter line used as a biomarker for Notch signaling activation) show ectopic activation throughout the ventricular endocardium (Figure 5C’, compare to 5C). This confirms the in situ data as well as previous reports that Prkd2 controls Notch signaling via HDAC regulation. Notably, Notch signaling remains unaffected in non-cardiac tissue (Figure 5B’ compare with B). These results reveal the critical role of Prkd2 in cardiac valve morphogenesis during early development.
2.5 PRKD2 and Calcineurin (CN) cooperate to regulate heart development

The Calcineurin/Nuclear Factor of Activated T-cells (CN/NFATc) signaling pathway plays a crucial role in cardiac valve morphogenesis (de la Pompa et al., 1998). Studies in embryonic mouse and fish heart valve remodeling link this pathway to the production of soluble factors such as vascular endothelial growth factor (VEGF) that regulate EMT (Timmerman et al., 2004; Gunawan et al., 2020). Since NFAT enters the nucleus and drives the transcription of genes involved in heart formation only when dephosphorylated, NFAT activation can result from either enhanced dephosphorylation (by activated calcineurin) or reduced phosphorylation (by a PKC/PRKD-dependent mechanism) (Prasad and Inesi, 2009). WT and heterozygous s411 mutant zebrafish embryo were treated with Cyclosporine (CsA), a Calcineurin inhibitor (under conditions that abrogate CN activity) to test the hypothesis that CN and PRKD2 cooperate to regulate zebrafish valve development. Figure 6A shows that WT CsA-treated embryos display heart defects nearly identical to those identified in s411 mutant embryos. Interestingly, heterozygous s411 embryos from an heterozygous cross showed enhanced sensitivity to CsA, displaying a phenotype at a CsA dose that does not affect wild-type embryos (2μg ml⁻¹) (Figure 6B,C). The observation that CsA treatment of WT embryos recapitulates the s411 Prkd2 T757A phenotype suggests that CN and PRKD2 act in a reciprocal manner to control the phosphorylation of signaling proteins that regulate valve development.

Finally, wild-type zebrafish embryos were treated with CID755673, an inhibitor of PRKD activity. While early treatment with the inhibitor led to severely dysmorphic embryos (implicating PRKD activity in overall embryo development), CID755673 treatment initiated at 30hpf resulted in the appearance of embryos that phenocopied the s411 morphology (outflow blood stenosis and blood regurgitation), (Figure 6D compare to 6A). These findings identify a specific time-window in which PRKD activity is required for cardiac development in zebrafish.

2.6 Translational implications for a Tbx5-Prkd2 axis

PRKD2 is significantly associated with coronary heart disease, but doesn’t reach the genome-wide threshold. It is interesting to note that the associated variant rs425105 is an eQTL for PRKD2 in various human tissues. There are two SNPs in perfect LD in the region (r2 = 1) rs425105 and rs60652743. The latter, rs60652743,https://pubs.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs60652743 may be of interest as it will affect TBX5 binding sites. Tbx5 encodes for a T-box containing transcription factor (TF), which plays a pivotal role in heart, eyes and forelimb

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development in many vertebrate species (Chapman et al., 1996; Gibson-Brown et al., 1998; Begemann and Ingham, 2000). Homozygous mutation of tbx5a in zebrafish embryos leads to lethal cardiac looping defects and impair of fin initiation and morphogenesis (also known as heartstrings) (Garrity et al., 2002). In order to evaluate the potential functional association between tbx5a and prkd2, we first examined whether prkd2 area in zebrafish contains Tbx5a TF binding sites (TFBS) using the Bio-tool of TFBS identification across species, ConTra v3 (Kreft et al., 2017). This database visualizes and identifies TFBS in any region surrounding a gene of interest. An analysis of a promoter region set 3000bp upstream and 300bp downstream of the first exon of prkd2 identified several sites correspond to Tbx5a DNA binding motifs (Figure 7A,B). Moreover, tbx5a morpholino injected embryos showed an earlier and more severe phenotype also in a s411 heterozygous incross, with tbx5a morphants exhibiting a failure of heart looping and absence of pectoral fin budding (Figure 7C, with the cardiac defects appearing earlier and being more severe when compared to the s411 mutants) and reduced prkd2 expression (Figure 7D). These results are consistent with the notion that Tbx5a regulates prkd2 expression.

3. Discussion

Heart valve defects are a leading cause of CHD, providing a strong rationale for studies that dissect the genetic and molecular factors that underlie heart valve formation. Zebrafish embryos survive early stages of embryo development without a functional cardiovascular system and therefore provide a particularly useful model to elucidate mechanisms of valve formation. This study uses a zebrafish model to identify the s411 mutant (that harbors a T757A substitution that inactivates Prkd2) with a complete outflow tract stenosis. Previous studies implicate a Prkd2-HDAC5 pathway in valve formation (Just et al., 2011). However, the observation that BMP signaling is impaired in s411 mutants and that s411 heterozygous embryos are hypersensitive to sublethal concentrations of the Calcineurin inhibitor cyclosporine would suggest that PKD regulates additional signaling pathways that control AV specification. Both PRKD1 and PRKD3 have been reported to exhibit a synergistic effect with the calcineurin signaling pathway to promote the expression of specific genes (Kim et al., 2008; Li et al., 2011). Although, it was demonstrated in vivo that PRKD1 stimulates myocyte enhancer factor-2 (MEF2) activity and it’s involved in pathological cardiac remodeling in mice (Fielitz et al., 2008), it was then studied in skeletal muscle a possible mechanism through which PRKD1 cooperates with calcineurin to drive MEF2 expression and
slow-twitch fiber phenotype (Kim et al., 2008). In addition, another study showed that PRKD3 is required for the NFATc4, Nkx2.5, and GATA4 expression while acting downstream of calcineurin-activated NFATc1 and c3 in pathological cardiac hypertrophy (PCH) model (Li et al., 2011). Therefore, the signaling cascade calcineurin-NFATc1/c3-PKD3-NFATc4 in the myocardium is proposed for the PCH model. Cardiac valve development is a very dynamic and complex morphogenetic process involving signals from the myocardium to the endocardium. For example at E9 in heart valve morphogenesis in mice, NFAT is required to repress VEGF expression in the myocardium. However later, at E11, a second wave of calcineurin/NFAT signaling is required in the endocardium, adjacent to the earlier myocardial site of NFAT action, to direct valvular elongation and refinement and is required for valve interstitial cell development (Chang et al., 2004). A conserved role for the second wave of Nfatc1 for VICs is recently also discovered in zebrafish (Gunawan et al., 2020). Our findings propose that a serine/threonine protein kinase (PRKD2) acts synergistically with a serine/threonine phosphatase (calcineurin) pathway to fine-tune heart development and function both in the myocardium and the endocardium plausibly also in several stages.

Moreover, PRKD2 expression has been correlated with key genes involved in Notch pathway in newly diagnosed acute myeloid leukemia (AML) patients (Liu et al., 2019) and there is evidence that PRKD2 promotes proliferation and chemo-resistance of human AML cell lines through a mechanisms involving Notch activity (Liu et al., 2019). Our observation that notch1b, bmp4, and the flow responsive transcriptional factor klf2a (factors that have been implicated in AV valve formation (Kalogirou et al., 2014) are detected throughout the hearts of s411 mutants - these factors do not localize to the AV region – reinforces the notion that PKD2 sits at a nodal point controlling a number of signaling pathways that regulate AV valve formation. In addition, another study has recently revealed a PRKD1-HDAC4/5-TBX5 regulatory pathway via PRKD1 relief of HDAC4/5-mediated post-translational suppression of TBX5 transcriptional activity (Ghosh et al., 2019). In our study, from an unbiased initial observation that a polymorphism in humans is expected to disturb a TBX5 binding site, we identified Tbx5 elements in the zebrafish promoter of prkd2 and then verified downregulation of prkd2 expression upon injection of a well-characterized tbx5 morpholino. The interaction of PRKD2 with TBX5 suggests that prkd2 is also regulated in the myocardium, a novel regulatory interaction in heart development, which warrants further investigation of the underlying mechanism.

The origin of congenital heart defects has been linked with the properties of cardiac progenitor cells and the mechanisms of their development. Myocardial specification and differentiation during the initial growth of the heart tube, its elongation and the cardiac looping
are well-orchestrated processes. In mammals, early cardiomyocytes that contribute to the formation of the left ventricle, atrioventricular canal and atria arise from a collection of progenitor cells named the first heart field (FHF). Subsequent addition of cardiomyocytes to the early cardiac tubule that forms the outflow tract, right ventricle, and inflow region occurs via a second distinct cell lineage termed the second heart field (SHF) (Buckingham et al., 2005; Rochais et al., 2009). Thus, the embryonic heart development occurs by cardiomyocytes deriving from two cell lineages corresponding to the contribution of HFH and SHF. Despite the difference of heart morphology between the two-chamber zebrafish heart and mammalian heart, it has been shown that similar regulatory networks drive the fish cardiac cell fate. In the fish model, FHF is the source of cells for the formation of the primitive heart tube, and cells derived from SHF are added to the heart tube and build structures at the inflow and outflow tract (De Pater et al., 2009; Hami et al., 2011; Musso et al., 2015). The discrete cardiomyocyte differentiation phases of zebrafish heart revealed that the two-cell lineage model is a conserved mechanism.

Studies have shown the critical role of cardiac neural crest cells in regulating the second heart field differentiation and that the absence of neural crest-derived mesenchyme in the pharyngeal impacts negatively on heart tube elongation. SHF cells contribute to the outflow tract and the structure and morphology of bulbus arteriosus in zebrafish. Several signaling pathways emerge in regulating this complex morphogenetic process where the heart and endocardium continues to the outflow tract endothelium, which is supported by smooth muscle cells. Pathways that play crucial role in atrioventricular development are emerging to be involved also in shaping the outflow tract. These include the Yap1 klf2a/Notch axis (Duchemin et al., 2019) as well as Tgfβ (Boezio et al., 2020). Based on that, it is not surprising that prkd2 mutants show defects both in the bulbus arteriosus (initially) and the atrioventricular valve. prkd2 knockout could cause a similar phenotype in mice as in s411 mutant zebrafish embryos, but conditional and inducible knockouts would be necessary in order to validate and study the respective murine phenotype.

In zebrafish the Prkd family consists of three members of (prkd1, prkd2, prkd3). prkd2 is located on chromosome 15, whereas both prkd1 and prkd3 are found on chromosome 17. Previous studies implicate zebrafish Prkd1 in the regulation of angiogenesis and lymphangiogenesis during development and as essential for tumor angiogenesis; silencing of prkd1 results in reduced formation of the intersomitic vessels and parachordal lymphangioblasts and abolished tumor angiogenesis (Hollenbach et al., 2013). Of note, while the overall homology of Prkd1, Prkd2, and Prkd3 is between 60-70%, their catalytic domains show 91% amino acid similarity. However, our identification of an inactivating Prkd2-T757A
substitution that drives the s411 mutant phenotype, argues that the Prkd family enzymes play non-redundant roles during zebrafish development. Finally, it also validates the unbiased forward genetic screen approach to identify important functional residues in proteins with key biological activity.

4. Materials and Methods

4.1 Zebrafish maintenance and breeding

Zebrafish embryos were raised under standard laboratory conditions at 28°C on a 14/10 h day/night cycle according to (Aleström et al., 2020). The following transgenic lines were used: Tg(kdrl:EGFP)s843 (endothelia/endocardial) (Jin et al., 2005), Tg(myl7:DsRed)s879 (myocardium) (Chi et al., 2008) and Tg(Tp1:mCherry) (Notch-responsive cells) (Ninov et al., 2012). s411 is identified in (Beis et al., 2005). Embryos were raised up to 120hpf. Therefore, these experiments are not animal experiments and do not fall under the protection guidelines of the directive 2010/63/EU revising directive 86/609/EEC on the protection of animals used for scientific purposes as adopted on 22 September 2010. Genotypic and adult handling of animals experimentations were approved from the Bioethics and Animal committees of BRFAA and the Veterinary department of Attica region (number 247916, 08/04/20) for facility EL 25 BIOexp 03. Embryos and larvae were anaesthetized for imaging by incubation in tricaine methanesulfonate, MS-222 (0.003%).

4.2 Genetic mapping, mutation detection

For the accurate physical positioning of the mutation, highly annotated zebrafish genetic maps (MGH, LN54, T51) and known microsatellite markers were used. Genetic lineage analysis facilitating markers of 30cM distance throughout the zebrafish genome initially mapped s411 on chromosome 15 (LG-linkage group15). Polymorphism marker linkage analysis on 865 embryos, restricted s411 mutation between the markers z6895 (28 recombinants out of 865 mutant embryos) and z51478 (23 recombinants out of 865 mutant embryos), while further fine-mapping located the mutation between markers on the overlapping BACs: CU062627 (1/28 recombinants) and BX924136 3/23 recombinants). Accordingly to the annotated physical map, all the candidate genes in this area were tested (prkd2, gpr184, 5s rRNA, urgcp and dscam-like). DNA from s411 and wild-type pooled embryos was sequenced of the open reading frames in the region verified a unique A to G mutation resulting to a T757A substitution.
4.3 Morpholino microinjection knockdown

Wild-type zebrafish embryos were injected at the one-cell stage with a splice blocking anti-prkd2 morpholino, designed to block the splicing of exon 11. cDNA sequencing of injected embryos verified the prediction and revealed that maintain exon 11 introduces a premature stop codon at amino acid 569. anti-prkd2 morpholino sequence is 5'-ATGTCAGGTCTTCATCTCCACAC-3'. anti-tbx5a morpholino sequence is 5'-TTCACGTGCCATGTGGAG-3' (Gene Tools, LLC).

4.4 Immunohistochemistry

The following primary antibodies were used at the indicated dilution: mouse monoclonal antibody zn5 at 1:10 (ZIRC), eln2 at 1:100 (gift from Prof. Frederick Keeley) (Miao et al., 2007). Wild-type and mutant zebrafish embryos at different time-stage (48, 72 and 96 hpf) were fixed overnight at 4°C or for 2 hours at room temperature in 4% paraformaldehyde. Whole-mount antibody staining was carried out in PBT (4% BSA, 0,3% Triton X in PBS, pH 7.3) overnight at 4°C. Stained embryos were embedded in 4% agarose/PBS (Sigma A9539-500G) and cut into 120-150 μm sections with a Leica VT1000S vibratome. Sections were covered by the antifading mounting medium, Vectashield (cat.No H-1000, Vector). Sections were incubated overnight with rhodamine phalloidin (Molecular Probes) at 1:500 in PBDT (PBS, 0.1% Tween, 1% DMSO) for filamentous actin staining.

4.5 RNA extraction and quantitative real-time PCR

Larvae were collected at the stage of 56hpf, euthanized, transferred in 2ml tube containing 300μl TRI Reagent (Sigma, T9424) and homogenized. Total RNA was extracted from the homogenized samples and reverse transcribed into cDNA using PrimeScript RT reagent kit (TaKaRa RR037a) according to manufacturers’ protocols, using 500 ng RNA starting material per cDNA synthesis reaction. qPCR reactions were analyzed on a Roche Lightcycler 96 (Roche Life Science) using KAPA SYBR FAST (Sigma-Aldrich, KK4611). Cycling conditions were as follows: 2 min at 50 °C and 10 min at 95 °C followed by two-step PCR for 40 cycles of 15 sec at 95 °C and 60sec at 60 °C. Relative expression of prkd2 was normalized to the average of the stably-expressed reference genes act2b and ef1a, and calculated 2^−ΔΔCt values are presented. The primers used are:

actin2b : Fw: 5'-CGAGCTTCTTCCCATCCA-3' Rev: 5'-TCACCAACGTAGCTTTTCT-3'
**4.6 Confocal microscopy**

Imaging was performed using Leica STP6000 and Leica TCS SP5 inverted confocal microscope. The images were captured and analyzed with the LAS AF software.

**4.7 In situ hybridization**

Whole-mount RNA *in situ* hybridization (ISH) using *notch1b*, *bmp4* and *klf2a* antisense probe was performed in embryos at 72 hpf, according to The Zebrafish Book (Westerfield, 2000).

**4.8 Plasmid generation**

Mutant forms of human PRKD harboring single residue substitutions were generated using the QuikChange mutagenesis system (Agilent Technologies) and then validated by sequencing. Expression vectors were introduced into HEK293 cells (maintained in Dulbecco’s modified Eagle’s medium [DMEM] with 10% fetal bovine serum [FBS]) using the Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. After 24 h, cells were lysed in RIPA buffer containing 1 mM sodium orthovanadate, 10 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ leupeptin, 10 μg ml⁻¹ benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin A, and 0.1 μM calyculin. Cell lysates were used for *in vitro* kinase assays with a recombinant human CREB-maltose binding protein fusion construct (from Biosource) included as substrate according to methods described previously. Immunoblot analysis to track PRKD autophosphorylation and heterologous substrate phosphorylation was performed with antibodies from Cell Signaling Technology according to standard methods and as described previously (Guo *et al.*, 2011).
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**Figures**

A

wt

B

s411

C

prkd2 MO

D

E

Wild-type  

s411 het  

s411 mutant

F

Zebrafish  

Homo sapiens  

Pan troglodytes  

Canis lupus familiaris  

Rattus norvegicus  

Mus musculus

G

Figure 1: **s411 carry a mutation in the prkd2 gene.** (A, B) Bright field image analysis of a wild-type zebrafish embryo compared to a s411 mutant embryo at 72hpf. Heterozygous adults that carry the s411 recessive mutation give 25% offspring mutant embryos exhibiting heart edema, inadequate blood circulation leading to complete outflow tract stenosis and blood regurgitation by 72hpf. Scale bar 500μm. (C) Bright-field image analysis prkd2-targeted morpholino-injected embryo at 72hpf. MO-prkd2-injected embryos resemble the s411 phenotype possessing the same features as s411 mutants (3 replicates, n>=50). Scale bar 200μm. (D) Bulked segregant analysis and further genetic mapping positioned the s411 mutation on chromosome 15. Recombination analysis on 865 embryos, positioned the mutation initially between the markers, z6895 and z51478 and further fine-mapping positioned the mutation between markers we developed in the overlapping BACs: CU062627 and BX924136. (E) The s411 embryos carry an A to G mutation that translates to a Threonine (T) to Alanine (A) amino acid change. (F) Blast analysis showed that the threonine T757 (T714 in Humans) is a conserved amino acid at a highly conserved region of the C-terminus PRKD2 kinase, between several species. (G) Schematic representation of zebrafish Prkd2 kinase. It consists of 923 amino acids and the main domains of the enzyme are indicating: two cysteine-rich motif
domains (cys1 and cys2), a pleckstrin homology domain (PH), and the C-terminal catalytic domain where the PKC-phosphorylation sites, Ser749 and Ser753 reside. It is also highlighted the position of s411 mutation (A-to-G), a previously identified zebrafish mutation with a similar phenotype (Y849) and the position of premature stop codon after MO injection resulting to defective splicing.
Figure 2: The T757A substitution disrupts PRKD2 kinase activity. Lysates from HEK293 cells that heterologously overexpress WT or mutant forms of PKD1 and PKD2 were subjected to in vitro immuno-complex kinase assays (IVKAs) in the absence or presence of PS/PMA and immunoblot analysis was used to track PKD C-tail autophosphorylation as well as PKD1 phosphorylation of recombinant CREB (added as a heterologous substrate). All results were replicated in three separate experiments.
**Figure 3**: *s411* embryos show aortic valve stenosis. (see also supplementary movies 2,3). Confocal images of the heart (A,A’,E,E’), the AV canal (B,B’), the outflow track (C,C’) and the bulbus arteriosus (D,D’) at 72hpf. *Tg (kdrl::EGFP)* (green) embryos were stained for F-actin (red) (A,A’) or zn5 (pseudo-colored red) and eln2 (pseudo-colored blue) (D,D’). *Tg(kdrl::eGFP) (green)/Tg(myl7:DsRed)* embryos were stained for zn5 (pseudo-colored blue) (C,C’, E,E’). (A,A’) In *s411* embryos, the myocardium appears thinner compared to the wild-type embryos. Arrows indicate the myocardial wall. (B,B’) Endocardial cells residing at AV canal extend in two layers at wild-type embryos whereas the *s411* mutants obtain a single-layer of AV endocardial cells. Arrowheads indicate the endocardial cells of AV. (C,C’) The endocardial cells of outflow track are in tight contact in *s411* mutant embryos. This structural feature leads to stenosis formation. Arrow indicates the junction between the neighboring endocardial cells. (D,D’) Bulbus arteriosus consists of endocardial cells surrounded by a layer of smooth muscle cells. *s411* mutants have a blocked bulbus arteriosus. Arrows show the elastin-positive cells and the stenosis of mutant embryos. (E,E’) In *s411* embryos, the endocardial
monolayer of AV cells does not express zn5 compared to the two-layer zn5+ AV endocardial cells in wild-type embryos, which indicates the malformation of atrioventricular cardiac valve during development. A, atrium; V, ventricle; AV, atrioventricular, BA, bulbus arteriosus. n=10 in each of 3 independent experiments. Scale bar 50μm for A,A’, E,E’ and 20μm for B-D’.
Figure 4: Atrioventricular canal markers remain expressed throughout the heart in \textit{s411} mutant embryos. Ectopic expression of atrioventricular canal (AV) markers in \textit{s411} mutant embryos as shown by whole-mount \textit{in situ} hybridization. \textit{notch1b} antisense probe in lateral (A,B) and dorsal (A',B') view of wild-type embryos (A,B) and \textit{s411} mutants, respectively (A',B') at 72hpf. \textit{klf2a} in wild-type (C) and \textit{s411} mutants (C') and \textit{bmp4} in wild-type (D) and \textit{s411} mutants (D') at 72hpf. All three AV differentiation markers show restricted expression pattern in the AV and outflow tract of wild type embryos, while they remain expressed throughout the heart in \textit{s411} mutants. \( n=10 \) in each of 3 independent experiments Scale bar 150\,\mu m.
**Figure 5**: Ectopic activation of endocardial Notch signaling in *s411* embryos carrying the T757A PRKD2 mutation. Bright field (A,A’) and fluorescence (B,B’) analysis of *s411* sibling and mutant embryos carrying the *Tg(Tp1:mCherry)* (pseudo-colored grey). Scale bar 500μm. Confocal analysis of 150 μm cardiac slices of *s411* sibling and mutant embryos carrying the *Tg(Tp1:mCherry)* co-stained with 633-phalloidin (blue) (C,C’); Scale bar 25μm. Notch signaling is active in several tissues and organs throughout the embryo and restricted to the AV canal and OFT of wild-type embryos at 72hpf (B,C). In *s411* mutants, Notch signaling appears unaffected.
in the embryo (B’ white arrowheads) but remains active throughout the ventricular endocardium (B’ yellow arrowheads, C’). White and yellow arrows (C) indicate the Notch-positive cells at AV canal and OFT, respectively. AV, atrioventricular; OFT, outflow tract n=10 in each of 3 independent experiments.
Figure 6: Disruption of calcineurin/NFATc signaling and blocking PRKD activity exhibits a similar phenotype to s411 mutant embryos. Incubation of wild-type (A) with 10μg ml⁻¹ cyclosporine A (CsA) results to a s411 phenotype. s411 heterozygous (B) embryos treated with cyclosporine A, at 2μg ml⁻¹, a sublethal dose of CsA show phenotype while there is no effect in wild-type embryos (C) when treated at this dose. (D) Treatment of wild-type embryos with a Protein Kinase D family inhibitor, 2,3,4,5 -Tetrahydro-7-hydroxy- 1H-benzofuro[2,3-c]azepin-1-one between 30-72hpf phenocopies s411 mutants showing that this is the critical window of prkd2 activity in the heart. n=20 in each of 3 independent experiments. Scale bar 100μm.
Figure 7: PRKD2 association with TBX5a regulation. Multiple predicted transcription factor Tbx5a binding sites in the promoter region of zebrafish Prkd2 via the ConTraV3 web server (A) and the possible DNA binding motif (B). Brightfield image analysis and quantification of 72hpf tbx5a-morpholino injected s411 siblings, tbx5a-morpholino injected wt and control wt embryos (C). tbx5-morpholino injected s411 mutant embryos exhibit an earlier more severe phenotype reminiscent to the tbx5 morphants and mutants; scale bar 100μm. As measured via rt-qPCR, prkd2 expression levels in tbx5 morphants are reduced compared to uninjected siblings at 56hpf, normalized both to act2b and elf1a as reference genes (D). n= 6 independent replicates, 15 larvae per sample, Student’s t-Test (two-tailed distribution, paired), ** significantly different p-value <0.01, error bars +SEM.
Movie 1. Brightfield video of wild-type (bottom) versus s411 (top) at 72hpf, showing the outflow tract stenosis at physiological speed.

Movie 2. Resonant scanner confocal movie of the outflow tract of wild-type Tg(kdrl::eGFP);Tg(gata1::DsRed) at 1/10 speed.

Movie 3. Resonant scanner confocal movie of the outflow tract of s411 Tg(kdrl::eGFP);Tg(gata1::DsRed) at 1/10 speed.