The Role of Ca2+/Calmodulin Dependent Protein Kinase II, CaMK-II, in Kidney Morphogenesis

Sarah R. Ingram
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The Role of Ca\(^{2+}\)/Calmodulin Dependent Protein Kinase II, CaMK-II, in Kidney Morphogenesis

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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April 2020
Acknowledgements

I would like to thank the many people who have helped me professionally and personally throughout this dissertation project. I would first like to thank Dr. Robert Tombes for giving me the opportunity to work in his lab and for supporting me through this project. In spite of his many obligations he always found the time to be a part of my project, encourage me to think about my work in different ways, and provide a boost of confidence whenever it was needed. I would also like to thank Dr. Sarah Rothschild for taking the time to teach me every technique I would use during this project, and for providing countless hours of support and guidance. Her shared joy in my successes and endless patience through my failures helped me know there was always someone in my corner cheering me on, and gave me the confidence to achieve my goals. I would also like to thank my undergraduate mentor, Dr. Christopher Ehrhardt, for instilling in me a love of research and for encouraging me to begin this journey. I would also like to thank my committee members Dr. Greg Walsh, Dr. James Lister, and Dr. Joyce Lloyd for providing their time and experience to help me grow as a researcher and for helping provide new ideas to help make this project successful.

I would like to express my gratitude to all of the members of the Tombes/Rothschild lab who have supported me throughout this project. I would like to thank all of the undergraduate researchers who have helped with this project, including Daniel Mohammadi, Jamie Parkerson, Mallory Downey, and Maddy Seputro. I would also like to thank my fellow graduate students Camden Kurtz, Sanyam Patel, and Alex Belt. They were always ready to be a sounding board for new ideas and always provided endless laughs when I needed it most. I am eternally grateful for their friendship during this project.

Finally, I would like to thank my friends and family. I would like to thank my friends Lauren Bibbs, Stephen Klimek, Jim Murphy, and Rebecca Andrus for being my support system throughout this journey. I would like to thank my parents, Donnie and Becky Wilkerson, and my step-mother, Tracy Wilkerson, for instilling in me the importance of education and always encouraging me to be the best that I could be. I would like to thank my sister, Mary Wilkerson, for being the most loving sister anyone could ask for. I would also like to thank my mother-in-law, Belinda Ingram, for always being there when I needed her, and for always believing in me. Most importantly, I would like to thank my husband, Ryan Ingram, for his support throughout this process, without whose love and patience I would never have made it this far.
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<td>ADPKD</td>
<td>Autosomal dominant polycystic kidney disease</td>
</tr>
<tr>
<td>ARPKD</td>
<td>Autosomal recessive polycystic kidney disease</td>
</tr>
<tr>
<td>ADPLD</td>
<td>Autosomal dominant polycystic liver disease</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium, ionized</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMK-II</td>
<td>Ca$^{2+}$/calmodulin dependent protein kinase II</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Palindromic Repeats</td>
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<tr>
<td>dpf</td>
<td>Days post fertilization</td>
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<tr>
<td>eflα</td>
<td>Elongation factor 1-alpha</td>
</tr>
<tr>
<td>GANAB</td>
<td>Glucosidase II subunit α</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilization</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HDAC4</td>
<td>Histone deacetylase 4</td>
</tr>
<tr>
<td>HDAC5</td>
<td>Histone deacetylase 5</td>
</tr>
<tr>
<td>Na$^{+}$/K$^{+}$ ATPase</td>
<td>Sodium potassium ATPase</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NMD</td>
<td>Nonsense Mediated Decay</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PKD</td>
<td>Polycystic kidney disease</td>
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<td>PKD1</td>
<td>Polycystic kidney disease 1</td>
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<td>PKD2</td>
<td>Polycystic kidney disease 2</td>
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<td>PKD3</td>
<td>Polycystic kidney disease 3</td>
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<td>PTU</td>
<td>1-phenyl-2-thiourea</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>RBP</td>
<td>RNA Binding Proteins</td>
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<tr>
<td>TALEs</td>
<td>Transcription activator-like effectors</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nucleases</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
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<tr>
<td>WISH</td>
<td>Whole mount <em>in situ</em> hybridization</td>
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Abstract
THE ROLE OF CA\textsuperscript{2+}/CALMODULIN DEPENDENT PROTEIN KINASE II, CaMK-II, IN KIDNEY MORPHOGENESIS

By Sarah R. Ingram, B.A.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2020

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Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common heritable diseases in the world, characterized by the development of large fluid filled cyst that inhibit kidney function. Treatments for this disease are currently limited due to a lack of understanding of disease pathogenesis. Ca\textsuperscript{2+}/calmodulin dependent protein kinase II (CaMK-II) is necessary for kidney morphogenesis in zebrafish as well as being a downstream effector of \textit{pkd2}, one of the genes most commonly mutated in ADPKD patients. The roles of CaMK-II during zebrafish kidney development include regulation of cell migration and pronephric convolution, as well as cloacal cilia stability. The influence of CaMK-II on these pathways is partially dependent on its regulation of HDAC4 localization. Inhibition of CaMK-II caused the translocation of HDAC4 from its location in the cytosol to the nucleus. This led to the model that CaMK-II activation via PKD2 retains HDAC4 in the cytosol allowing for transcription of its target genes. Notch signaling proteins were also identified to be potential members of this pathway as inhibition of CaMK-II caused a reduction in notch signaling in the cloaca as well as an increase in expression of the notch ligand \textit{deltaC}. This increase in \textit{deltaC} expression in embryos with inhibited CaMK-II was rescued by suppression of \textit{hdac4}. Further studies attempted with a zebrafish \textit{camk2g1}
mutant proved inconclusive as mutants displayed none of the phenotypic defects seen in morphant embryos. Analysis of CaMK-II gene expression in camk2g1 mutants identified the capability of genetic compensation between CaMK-II isoforms. In camk2g1 mutants, the paralog gene camk2g2 is upregulated over 3 fold, compensating for the loss of its paralog gene, thereby preventing the phenotypic defects typically seen in camk2g1 morphants. This compensation is not immediate as upregulation of camk2g2 was not seen in G0 camk2g1 CRISPR embryos. In conclusion, this study has not only furthered the understanding of the roles of CaMK-II during kidney development and identified new potential therapeutic targets for ADPKD patients, but it has also demonstrated the ability of CaMK-II isoforms to compensate for one other. In addition to providing a greater understanding of the relationship between CaMK-II paralogs, this study also further validates the uses of knockdown methods in developmental studies as compensation was found not to occur in these models.
Chapter 1: Background

Polycystic Kidney Disease

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is one of the most common heritable diseases in the world (Igarishi and Somlo, 2002). In spite of its prevalence, no specific treatments exist due to a lack of understanding of its pathogenesis (Igarishi and Somlo, 2002). ADPKD is characterized by the development of large fluid-filled kidney cysts that gradually enlarge over time and replace healthy renal tissue (Igarishi and Somlo, 2002) (Kottgen, 2007). Cystic development can also occur in other organs such as the pancreas, liver, spleen, and ovaries (Gabow, 1993). This disease is thought to affect anywhere from 1 in 400 to 1 in 1000 individuals and is the fourth leading cause for renal replacement worldwide (Ong et al., 2015).

Initiation of cyst development in ADPDK patients requires a primary germline mutation in genes PKD1 and/or PKD2, as well as a secondary somatic mutation in either PKD1/PKD2 (Igarishi and Somlo, 2002). A PKD3 gene has been proposed in order to account for patients lacking mutations in PKD1 or PKD2 (Porath et al., 2016). A candidate for this gene is GANAB, which encodes for glucosidase II subunit α (Porath et al., 2016). Mutations in this gene have been shown to cause mild ADPKD and mild to severe autosomal dominant polycystic liver disease (ADPLD) (Porath et al., 2016). However, mutations in GANAB only account for 3% of unresolved cases, implying that other genes are involved in ADPKD development in patients without mutations in PKD1 and PKD2 (Porath et al., 2016).

PKD1 codes for polycystin-1, a conserved membrane protein that is able to interact with a variety of other proteins such as lipids and carbohydrates (Wilson, 2006). PKD1 contains domains known to be involved in cell-cell and cell/matrix interactions (Chauvet et al., 2002). Expression of PKD1 can be seen in human fetal tissue samples in the neural tube, cardiomyocytes,
mesonephros, and endodermal derivatives (Chauvet et al., 2002). PKD1 expression can also be seen in differential proximal tubules during nephron development. Although many of the roles of PKD1 are still being elucidated, it is thought to have roles in transcription factor activation, G-protein binding, regulation of Wnt signaling, as well as regulation of proliferation and resistance to apoptosis (Parnell et al., 1998) (Arnould et al., 1998) (Kim et al., 1999) (Boletta, 2000).

Approximately 85% of ADPKD cases are caused by mutations in PKD1 (He et al., 2011). These mutations can occur in a variety of forms including nonsense mutation, frame shift mutations, missense mutations, and aberrant splicing (He et al., 2011). It is thought that patients with mutations in the 5’ regions suffer from a more severe form of ADPKD than those with mutations in the 3’ region (He et al., 2011). Homozygous knockout of Pkd1 in mice causes severe renal cyst development and death by embryonic day 15 (He et al., 2011). Heterozygous mutants for Pkd1 are viable. However, they still exhibit renal and hepatic cyst development (He et al., 2011) (Piontek et al., 2004). Loss of Pkd1 also causes other defects in mice including cardiovascular and skeletal defects (Boulter et al., 2001) (Kim et al., 2000).

PKD2 encodes for polycystin-2 (TRPP2), a member of the TRP family of Ca^{2+} ion channels (Chauvet et al., 2002). PKD1 forms a complex with PKD2 enabling the formation of a non-selective cation channel (Clapham 2003) (Mochizuki et al., 1996). In human tissue, PKD2 can be found in the plasma membrane, endoplasmic reticulum, and the primary cilium and has various functions depending on its location within the cell (Igarishi and Somlo, 2002) (Cai et al., 1999). In the plasma membrane, when complexed with PKD1, PKD2 functions as a non-selective cation channel with a higher selectivity for Ca^{2+} (Hanaoka et al., 2000) (Tsiokas et al., 2007). In the endoplasmic reticulum, PKD2 functions as an intracellular Ca^{2+} channel that is activated in response to alterations in intracellular Ca^{2+} levels (Cai et al., 1999) (Tsiokas et al., 2007). In the
primary cilium, \( PKD2 \) acts as a regulator of intracellular \( \text{Ca}^{2+} \) in response to fluid flow (Tsiokas et al., 2007) (Praetorius and Spring 2001). \( PKD2 \) has also been found in the mitotic spindle and centrosomes (Tsiokas et al., 2007). Although its exact function at these locations is not completely known, it is thought that it could be responsible for regulating \( \text{Ca}^{2+} \) levels during cell division (Rundle et al., 2004).

Mutations in \( PKD2 \) account for approximately 15\% of ADPKD cases (He et al., 2011). Typically, patients with mutations in \( PKD2 \) have a smaller number of cysts and don’t exhibit other symptoms until later in life. However, patients with mutations in \( PKD2 \) still suffer from a high frequency of renal failure with advanced age (Stypmann et al., 2007) (Hateboer et al., 2007). Homozygous and heterozygous mouse mutants for \( Pkd2 \) develop cysts in the kidneys and liver (Wu et al., 1998). In addition to cyst development, mouse mutants for \( Pkd2 \) also exhibit loss of left-right asymmetry, defects in reproductive tract development, and cardiovascular defects, a condition often seen in patients (Kuol et al., 2016) (Wu et al., 2000) (Pennekamp 2002) (Nie et al., 2014).

**Zebrafish (Brachydanio rerio) as a Model Organism**

The use of Brachydanio rerio, more commonly known as zebrafish, as a model organism originated in the 1970s with the work of George Streisinger. Zebrafish are a small freshwater cyprinoid fish that is thought to have originated in India, and can now be commonly found in pet stores around the world. Streisinger and his colleagues recognized the advantages of using zebrafish as a model for developmental and genetic studies, including their short generation time, high fecundity, external fertilization, and transparency during development (Streisinger et al., 1981). Through their work using zebrafish, Streisinger and his lab were able to develop protocols
for using zebrafish for a variety of applications including cloning (Streisinger et al., 1981), mutagenic studies (Grunwald et al., 1988) and cell lineage tracing (Streisinger et al., 1988).

Since the work of Streisinger and his lab, zebrafish have become a staple in scientific research around the globe. The Sanger Institute started the zebrafish genome sequencing project in 2001 and has since created a full reference genome. (Howe et al., 2013). Zebrafish contain 25 chromosomes (Postlethwait et al., 2000) and the zebrafish genome is thought to contain 26,206 protein-coding genes (Howe et al., 2013). Of those protein-coding genes, approximately 70% are believed to have a human orthologue (Howe et al., 2013). The similarities between human and zebrafish genomes make the zebrafish a unique and essential tool for human medical research.

Zebrafish provide an excellent model to study kidney development due to common cell types and molecular pathways as well as their transparency during early development (Drummond and Davidson, 2010). During embryogenesis, zebrafish possess a pronephric kidney which consists of two pronephric ducts fused at the proximal end with the glomeruli and fused at the distal end with the cloaca (Drummond and Davidson, 2010). In zebrafish, kidney cells are specified from the intermediate mesoderm at approximately 18hpf. The cells then undergo epithelialization at approximately 24hpf before migrating anteriorly via collective cell migration where they turn inward to connect with the glomerulus (Drummond and Davidson, 2010) (Vasilyev et al., 2009) This turning inward to connect with the glomerulus is known as ductal convolution and is thought to result from the piling up of cells at the anterior end (Vasilyev et al., 2009). Angiogenesis then occurs and the pronephros are fully functional by 48hpf (Drummond and Davidson, 2010).
Zebrafish Models of Polycystic Kidney Disease

Zebrafish have proven to be a useful model for ADPKD caused by mutations in both *pkd1* and *pkd2*. Zebrafish models for *pkd1* loss of function exhibit many defects in development. Inhibition of zebrafish paralogs *pkd1a/pkd1b* caused alterations in extracellular matrix proteins, hydrocephaly, craniofacial defects, and pronephric cyst development in a low percentage of embryos (10-15%) (Mangos et al. 2010). Zebrafish with a loss of *pkd1* also showed lymphatic system defects and cardiac edema by 5 days post fertilization (Coxam et al., 2014).

Zebrafish models of ADPKD resulting from loss of *pkd2* expression have also proven to be a valuable tool in understanding disease traits and progression. Like other models, downregulation of *pkd2* results in the development of kidney cysts and defects in left-right asymmetry (Mangos et al., 2010) (Obara et al., 2006) (Schottenfeld et al., 2007). Other defects seen in zebrafish with a loss of *pkd2* include hydrocephaly as well as body curvature, which is thought to result from alterations in the extracellular matrix (Mangos et al., 2010) (Obara et al., 2006). Zebrafish injected with *pkd2* mRNA that lack either the C-terminus or N-terminus dimerization domain also resulted in the formation of kidney cysts (Feng et al., 2008).

Calcium Calmodulin Dependent Protein Kinase II (CaMK-II)

CaMK-II is a serine/threonine protein kinase encoded by four genes in eukaryotes (α, β, γ, δ) (Tombes et al., 2003). CaMK-II forms oligomers of up to 12 subunits of which the subunits can be the same or different isomers (Bayer and Schulman, 2019). Each monomer of the holoenzyme consists of a catalytic, variable, and association domain (Tombes et al., 2003). The catalytic domain is 315 amino acids in length and contains the CaM binding domain. The variable domain can be between 30-100 amino acids in length and is subject to alternative splicing. The association domain is 135 amino acids in length and is responsible for the oligomerization of the holoenzyme
The formation of the holoenzyme brings catalytic heads into proximity, facilitating the unique ability of CaMK-II to autophosphorylate at site T^{287}. CaMK-II is activated by the binding of Ca^{2+}/CaM binding which relieves inhibition of the autoinhibitory arm, allowing for phosphorylation of site T^{287} (T^{286} in α). (Hudman and Schulman, 2002). The kinase is then capable of autophosphorylating the other subunits of the holoenzyme allowing for it to remain up to 80% activated after calcium stimulus has subsided (Tombes et al., 2003) (Rosenberg et al., 2005).

CaMK-II is best known for its role in the central nervous system. It comprises over 1% of total protein in the brain, where it is a known regulator of neuronal plasticity and cognitive function (Bayer and Schulman, 2019). It is not only a key protein involved in long term potentiation, a process correlated to the formation of memories, but has recently been identified as a regulator of long term depression as well (Wang et al., 2013) (Hudman and Schulman, 2002) (Bayer and Schulman, 2019). Mice lacking αCaMK-II, the predominate form of CaMK-II in the brain, showed diminished long term potentiation in the hippocampus (Hinds et al., 1998). Conversely, mice that overexpressed activated CaMK-II had impaired context discrimination (Ye et al., 2019). These studies along with many others over the years have demonstrated the importance of CaMK-II in the brain.

In addition to its roles in the brain, CaMK-II has also been shown to be present in almost every tissue and is involved in a large variety of processes throughout the body (Tombes et al., 2003). CaMK-II is involved in Ca^{2+} regulation in the heart as well as heart morphogenesis (Maier and Bers, 2002) (Rothschild et al., 2009). It also has a role in regulating cell motility by inducing de-phosphorylation of focal adhesion proteins thereby promoting focal adhesion turnover. (Easley et al., 2008). CaMK-II is also involved in left-right asymmetry in vertebrates as well as the
development of neurons in the enteric nervous system (Langerbacher and Chen, 2008) (Francescatto et al., 2010) (Gao et al., 2012).

**CaMK-II as a PKD2 Target**

Using zebrafish as a model organism, CaMK-II was determined to be required for kidney morphogenesis (Rothschild et al., 2011). CaMK-II is encoded by 7 genes in zebrafish (α, β1, β2, γ1, γ2, δ1, δ2) and is expressed throughout the embryo (Rothschild et al., 2011) (Rothschild et al., 2009). CaMK-II can be seen in the developing kidney beginning at 24hpf and is concentrated in the apical regions (Rothschild et al., 2011). Later, it can be seen throughout the pronephros at 30hpf at apical and basolateral cell surfaces as well as in intracellular clusters (Rothschild et al., 2011). Autophosphorylated CaMK-II is also present in the cloacal cilia at 30hpf (Rothschild et al., 2011). *Camk2g1* was shown to be the predominant form of CaMK-II present in the kidney (Rothschild et al., 2011).

*Camk2g1* expression was suppressed in zebrafish embryos using a translation blocking antisense morpholino oligonucleotide (*camk2g1 MO*), causing defects in kidney development along with several other deformities (Rothschild et al., 2011). These defects include pronephric cyst development, hydrocephaly, loss of left-right asymmetry, and body curvature (Rothschild et al., 2011) (Francescatto et al., 2010). Inhibition of *camk2g1* also caused a decrease in the distance kidney cells migrated during development (Rothschild et al., 2011). These cells were unable to undergo anterior convolution, preventing them from connecting with the glomerulus (Rothschild et al., 2011). This inhibition of kidney function was also demonstrated using a renal filtration assay showing a lack of fluid excretion over a 16 hour time period (Rothschild et al., 2011).

Inhibition of *camk2g1* caused specific defects in the distal region of the pronephric ducts. Pronephric occlusions were seen in the distal portion at 3dpf in 73% of morphants embryos. In
addition, cloacal cilia formed normally in camk2g1 morphants at 24hpf, but by 48hpf they had begun to destabilize, causing a reduction the number of cloacal cilia present from 13 in wild type embryos to one in camk2g1 morphants. The remaining cloacal cilia present in morphant embryos were found to be shorter and immotile (Rothschild et al., 2011).

A predominant defect seen in camk2g1 morphants was cyst development (Rothschild et al., 2011). This could be seen using both a camk2g1 morpholino and a dominant negative (DN) form of CaMK-II (K^{43}A) which lacks phosphotransferase ability. These defects in kidney development, as well as other associated defects like body curvature and hydrocephaly, mimic the phenotype of zebrafish embryos with inhibited pkd2 expression (Mangos et al., 2010) (Schottenfeld et al., 2007) (Feng et al., 2008) (Rothschild et al., 2011). This led to the model that Ca^{2+} entry via pkd2 enables activation of CaMK-II which in turn influences kidney development by regulating cellular migration, pronephric convolution, and cloacal cilia stability (Rothschild et al., 2011). This model was supported by the co-injection of the morpholino for pkd2 and a constitutively active (CA) form of CaMK-II (T^{287}D) which was able to rescue anterior migration and kidney convolution as well as reduce pronephric occlusions and hydrocephaly (Rothschild et al., 2011).
Chapter 2: Calcium Signals Act through Histone Deacetylase to Mediate Pronephric Kidney Morphogenesis

Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is the most common heritable disease in the world, occurring in approximately 1-400 to 1-1000 live births. This disease, characterized by the development of large fluid filled cyst, is caused by mutations in genes PKD1 and PKD2. PKD2 is a TRP cation channel necessary for Ca$^{2+}$ entry and activation of CaMK-II, a serine/threonine protein kinase required for kidney morphogenesis and cilia stability. In an effort to further elucidate the molecular pathways involved during kidney morphogenesis as well as identify potential therapeutic targets for ADPKD patients, the role of class II HDACs present during kidney morphogenesis was evaluated as well as their relationship with camk2g1, the predominate form of CaMK-II present in the pronephros. Class II HDAC family members (HDAC4, 5, and 6) were suppressed using antisense morpholino oligonucleotides and pharmacological inhibitors. While inhibition of hdac6 caused no gross morphological defects, inhibition of hdac5 resulted in the development of anterior kidney cysts and destabilized cloacal cilia, mimicking the phenotype of both pkd2 and camk2g1 morphants. Inhibition of hdac4 caused elongated cloacal cilia when compared to control embryos. The reduction of hdac4 or hdac5 activity in camk2g1 morphants caused opposing results. Suppression of hdac4 in embryos with reduced camk2g1 activity rescued cilia shorting and ductal convolution defects while suppression of hdac5 caused severe branching of the pronephros and a significant loss of pronephric cell migration. Further investigation into the relationship between class II HDACs and CaMK-II revealed that inhibition of camk2g1 caused a translocation of hdac4 from the cytosol into the nucleus. The subcellular localization of hdac5 was
unaffected by the loss of camk2g1 activity. These results suggest a role for CaMK-II in retaining HDAC4 in the cytosol, enabling primary cilia formation and anterior kidney morphogenesis.
Introduction

Histone Deacetylases

Histone deacetylases (HDACs) are conserved enzymes that catalyze the removal of an acetyl group from lysine residues. This most commonly occurs on histones, where removal of the acetyl group condenses the chromatin structure, preventing transcription of target genes (Seto and Yoshida 2014). Histone deacetylases also have a variety of non-histone targets where they act to regulate a number of cellular processes. These processes include ubiquitination and degradation of p53, deacetylation of α-tubulin, and regulation of cell motility (Luo et al 2000) (Hubbert et al 2002) (Di Giorgio et al 2013). The wide range of HDAC target was demonstrated using pan HDAC pharmacological inhibitors which caused an increase in acetylation in 10% of over 3500 tested acetylation sites (Choudhary et al 2009).

Histone deacetylases are divided into four classes based upon sequence similarity. Class I HDACs include HDACs 1, 2, 3, and 8. Class II consists of HDACs 4, 5, 6, 7, 9, and 10. Class IV consists of HDAC11 (Seto and Yoshida, 2014). These three classes are all part of the arginase/deacetylase superfamily whose targets include both histone and non-histone proteins. Class III consists of NAD+ dependent proteins SIRT 1-7 and belongs to the Sir2 regulator family which is involved in transcription silencing (Seto and Yoshida, 2014) (Brachmann et al., 1995).

Histone deacetylases are known to have roles in a variety of human diseases, making them an important candidate for therapeutic treatments (Seto and Yoshida, 2014). HDACs are associated with a variety of cancers and increased levels are associated with poor prognosis in ovarian and endometrial cancers (Li and Seto, 2016) (Weichert et al, 2008). In other types of cancers, such as melanoma and lung cancer, it is the loss of HDAC activity that is associated with poor prognosis.
(Li and Seto, 2016). Although their roles in various cancers may be complex, the use of selective and non-selective HDAC inhibitors have proven effective in the treatment of a number of cancers with over 20 pharmacological inhibitors being used in clinical trials (Li and Seto, 2016). HDAC inhibitors have also been successful in treating Alzheimer’s disease, where suppression of Hdac2 resulted in increased synaptic plasticity and memory formation in mouse models (Graff et al., 2012). HDACs have also been implicated in lung disease, cardiac abnormalities and a number of other disease making them an important topic of studies for treatment of human conditions (Seto and Yoshida 2014).

**Class II HDACs and CaMK-II**

Class II HDACs regulate MEF2C gene transcription, genes that encode for Ca$^{2+}$ dependent mediators of transcription (McKinsey et al, 2002). Interestingly, Mef2c knockout mice develop kidney cysts, mimicking the phenotype of both Pkd2 knockout mice and γ1 CaMK-II inhibited zebrafish (Xia et al., 2010) (Wu et al., 1998) (Rothschild et al., 2011). Interestingly, downregulation of HDACs in Pkd2 mutant mice have been able to inhibit cyst development (Xia et al, 2010). Two specific class II HDACs have a unique relationship with CaMK-II. HDAC4 possesses a CaMK-II specific docking site allowing it be directly phosphorylated by CaMK-II. This causes its translocation from the nucleus to the cytosol in muscle tissue (Backs et al, 2006) (Little et al 2007).

CaMK-II is also able to affect the localization of HDAC5 through direct interaction with HDAC4 (McKinsey et al, 2002). While HDAC5 does not possess a specific binding site for CaMK-II, it is able to hetero-oligermerize with HDAC4 through their respective coil-coiled domains. This allows for HDAC5 to become responsive to CaMK-II in two different way. One being by bound to HDAC4 while it is interacting directly with CaMK-II. The second way by a
transphosphorylation event when interactions between HDAC4 bringing it into close proximity to CaMK-II (Backs et al., 2008). The ability of CaMK-II to directly affect the subcellular localization of specific class II HDACs combined with their previously studied role in kidney cyst development in mice demonstrate a potential role for class II HDACs in polycystic kidney disease pathology. Through the model organism zebrafish, this study further explains the roles of HDACs during kidney development as well as demonstrates interactions between class II HDACs and CaMK-II, a protein known to be affected in zebrafish ADPKD models (Rothschild et al, 2011).
Methods and Materials

Zebrafish Strains and Care

Zebrafish embryos were obtained through natural matings and raised at 28.5°C (Kimmel et al., 1995). Zebrafish strains used were Wild-type (AB and WIK), Tg(atp1a1a.5:EGFP), and Tg(cmcl2:EGFP).

Fluorescent Localization

Embryos were fixed in 4% paraformaldehyde or Dent’s fixative as previously described (Rothschild et al., 2011). Embryos were then incubated with one of the following primary antibodies: rabbit anti-acetylated histone H3 (Millipore [06-599] at 2.5 µg/ml), mouse anti-acetylated α-tubulin (Sigma Chemical Co. [T7451] at 2 µg/ml), or mouse anti-α 1 Na+/K+-ATPase (Developmental Studies Hybridoma Bank, University of Iowa [α6F] at a 1:10 dilution). Embryos were incubated overnight in primary antibody after which they were placed with one of the following secondary antibodies: goat anti-mouse Alexa488 or goat anti-rabbit Alexa568 (Invitrogen at 2.5 µg/ml). In order to analyze craniofacial development, embryos were incubated overnight at 4°C with rhodamine phalloidin (Life Technologies, 1:1000). Embryo heads were then dissected and mounted on chamber slides, and imaged using confocal microscopy. Embryos were imaged either on a Nikon E600 compound microscope using a 20X dry, 40X dry, or 100X oil immersion objective, or on Nikon AZ-100 Macro Zoom fluorescent stereo microscope.
Morpholino Injections

Translation-blocking antisense morpholinos were designed to target zebrafish *camk2g1* (1ng), *hdac4* (2ng), *hdac5* (2ng), and *hdac6* (8ng) and were purchased from Gene Tools along with a control mismatch morpholino (2ng). All morpholinos were used as previously described with an injection volume of 1nl (Francescatto et al., 2010) (Rothschild et al., 2011) (Rothschild et al., 2013). The morpholinos for *hdac4*, *hdac5*, and *hdac6* were designed to target the following the regions around the translation start site: *hdac4*: CGCCACACTCACATCAACCATCAGC; nucleotides -11 to +14), *hdac5*: GATCTTCCCTGCCTGAAGTTCTCCA; nucleotides -38 to -14), *hdac6*: TTTGGTATCTGGAACCGCATCCATC; nucleotides -5 to +20).

cDNA Injections

Wild type zebrafish embryos were collected and RNA was extracted using TRIzol (Invitrogen) at 1, 6, 19, 24, 48, and 72 hours post fertilization. cDNA was generated using SuperScript III. *Hdac4* (sense: 5′-CCAAAGATGACTTCCCAGCTCCGC-3′, antisense: 5′-GAGGCTCTTTTGAATGTGACCC-3′), *hdac5* (sense: 5′-GGAACACCACCCTCCTACAAACTCC-3′, antisense: 5′GGAGGCTGTCTTGGAAGATCCGTCC-3′), and *hdac6* (sense: 5′-CCGCTTTGACTGCACGCTATGCT-3′, antisense: 5′-CTAAGAGAGCTCCAGAAGGGGGCGTG-3′) were amplified using High Fidelity Taq (Invitrogen). Amplified products were then cloned into the StrataClone vector (Agilent), and sequenced as previously described (Rothschild et al., 2007).
**Whole Mount In Situ Hybridization**

Digoxigenin-labeled antisense riboprobes were synthesized as previously described using T3 or T7 RNA polymerase (Rothschild et al., 2007). Embryos used for whole-mount in situ hybridization (WISH) were developed using alkaline phosphatase–conjugated anti-digoxigenin. Probes for Hdac4, hdac5, and hdac6 were designed to correspond to sequences amplified using the primer sets listed above. Cdh17, pax2a, and cmlc2 probes were prepared as previously described (Wingert et al., 2007; Wingert and Davidson, 2008).

**Mouse HDAC5 Expression Vector**

The murine hdac5 ORF was purchased from GE/Dharmacon (Clone ID: 5720438) and PCR-amplified using the following primers: Sense: 5′ GCGTCCGGAATGAACTCTCCCAACGAGTCGG 3′; Antisense: 5′ CGCTCTAGAGGGATGGGGGCCAGGGTGTCACAG 3′. It was then digested with BspEI and XbaI, and cloned into the pEGFP-C1 vector with N-terminal EGFP. This hdac5 construct was injected at the one cell stage into zebrafish embryos at a concentration of 30 ng/µl. The murine hdac4 was a gift from Dr. Eric N. Olson and was injected at 30 ng/µl at the one cell stage (Backs et al., 2006). HDAC4 and HDAC5 localization was assessed in live embryos at 24–48 hpf.

**Zebrafish HDAC4 Expression Vector and mRNA Synthesis**

Zebrafish HDAC4 was PCR-amplified (CGCGGATCCCATGACTTCCCACAGCCGTCCAG, GCGCTCGAGGATGGAGATTAGAGGACATCAG) from 24hpf cDNA and cloned into
the pCRII-TOPO vector (Invitrogen). HDAC4 was cloned into the Gateway middle entry vector using BamHI and XhoI. Cherry was amplified (CCAAGCTTATGGTGAGCAAGGGCGAGGA GG, GGATCCCAGCGCTTGTACAGCTCGTCCATGCC), digested with HindIII and BamHI, and ligated into HDAC4 entry vector. This vector was cloned into the final Gateway vector with the 5’ Sp6 pEntry vector and 3’ poly A entry vector. The construct was linearized using SpeI and mRNA was generated using the Sp6 mMMessage kit (Thermo)

**HDAC Rescue**

Hdac4 MO (2ng) and hdac4 RNA (100ng) were co-injected or sequentially injected into zebrafish embryos at the one cell stage. Embryos were assessed at 24hpf to assess left-right asymmetry. Embryos were fixed at 72hpf, labeled with mouse anti-acetylated α-tubulin in order to assess cilia length and number, and imaged using confocal microscopy.

Hdac4 MO (2ng) was injected into zebrafish embryos at the one cell stage alone or co-injected with mouse EGFP-HDAC5 (30ng/µl). Left-right asymmetry was assessed at 24hpf. At 72hpf, embryos were fixed and labeled with mouse anti-α1 Na+/K+-ATPase. Embryos were then analyzed for ductal convolution and anterior cell migration using confocal microscopy.

**Drug Treatment**

Zebrafish embryos were incubated in 0–200 nM TSA or 0–10 µM tubastatin A in 3 ml of system water starting at cleavage stage and continuing until fixing. Rescue experiments were performed
by continuously incubating \textit{camk2g1} morphants embryos with 100 nM TSA beginning at bud stage until being fixed.

\textbf{Generation of Nuclear and Cytosolic Targeted HDAC Constructs}

Point mutations were generated on the previously used EGFP-HDAC5 and Cherry-HDAC4 expression constructs using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies #200519). The mutated sites for HDAC4 were S458A, S606A, S458D, and S606E. The mutated sites for HDAC5 were S251A, S480A, S251D, and S480D. The construct was sequenced to confirm the mutation was achieved as well as to confirm no additional mutations were present. Products were then purified and transformed as previous described (Rothschild et al, 2011).

\textbf{Video Microscopy}

Live embryo cilia videos were obtained using DIC optics on a Nikon 60X water-immersion Plan APO objective and 20-frames-per-second acquisitions. Embryos were anesthetized and immobilized as previously described (Rothschild et al., 2011).

\textbf{Statistical Analysis}

Statistical significance was set at P<0.005 using the two-tailed \textit{t}-test
Results

**Expression of Class II HDAC 4, 5, and 6 are Detected in Zebrafish Embryos Throughout Early Development**

Three Class II HDACs were evaluated for their effects on early development based upon their previously established roles in relation to CaMK-II and kidney development. HDAC4 is known to be CaMK-II responsive in muscle tissue as well as possess a specific CaMK-II docking site (Di Giorgio and Brancolini, 2016). HDAC5 has also been shown to be responsive to CaMK-II, however only in the presence of HDAC4 (Backs et al., 2008). In contrast to other HDACs, HDAC6 deacetylates tubulin over histones and suppression of HDAC6 caused a reduction in cyst formation in mouse models of cystic kidney disease (Cebotaru et al., 2016; Li et al., 2016; Yanda et al., 2017a; Yanda et al., 2017b).

Expression of *hdac4*, *hdac5*, and *hdac6* can be seen using both semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) as well as *in situ* hybridization (Figure 2.1 U-Z). Transcript levels for all three genes were seen throughout development, from maternal transcripts through to 72hpf. All three transcripts were also able to be amplified from isolated kidneys at 24hpf. *Hdac4* and *hdac6* mRNA were also able to be amplified from isolated heart tissue at 36 hpf (Figure 2.1 U-W).

**Inhibition of Specific Class II HDACs Causes Defects in Early Development**

In order to evaluate the roles of HDAC4, HDAC5, and HDAC6 in early development, translation blocking antisense morpholinos were designed for each, and injected into zebrafish embryos at the 1-4 cell stage. Tail curvature defects can been seen in all three morphants at 24 hpf when compared to control embryos (Figure 2.1 A-D). These defects were persistent and could be
seen at 48hpf and 72hpf in all three morphants (Figure 2.1 E-L). Previous studies indicated a role for *hdac4* in craniofacial development in zebrafish embryos. Similarly, craniofacial defects, including clefting were observed in *hdac4* morphants at 72hpf, consisted with previously published data (DeLaurier et al., 2012) (Figure 2.1 M,N).

The effectiveness of the *hdac4* and *hdac5* morpholinos were assessed by examining the levels of acetylated histone 3 in morphant embryos at 48hpf. Acetylated histone 3 levels were elevated in morphants when compared to controls demonstrating a decrease in levels of *hdac4* and *hdac5* activity. The effectiveness of the *hdac6* morpholino was examined by observing acetylation levels in α-tubulin in lateral line axons and in the brain in *hdac6* morphants and control embryos. *Hdac6* morphants show an increase in acetylation levels in α-tubulin when compared to control embryos demonstrating a decrease in *hdac6* levels in morphants embryos (Figure 2.1 O-T).

At 48hpf, *hdac4* and *hdac6* morphants exhibited no defects in kidney morphology. However, *hdac5* morphants exhibited kidney cyst in the anterior portion of the pronephric ducts near the glomerulus (Figure 2.2 A-D). This mimics cyst development seen in both *camk2g1* morphants and *pkd2* morphants (Rothschild et al., 2011). This cyst development could be associated with lack of ductal convolution in these embryos. Ductal convolution is the turning inward of the two pronephric ducts in order to connect with the glomerulus. When ductal convolution was assessed in all three morphants, there was an absence of convolution in 80% of *hdac5* morphants. In contrast, only 15% of *hdac4* morphants and 3% of *hdac6* morphants failed to convolution (Figure 2.2 E-M, p<.005). This lack of convolution could be caused by a decrease in the distance pronephric cells migrate during development. This distance of cell migration can be examined by measuring the distance from the base of the ear and the top of the kidney at 72hpf (Rothschild et al, 2011). Compared to control embryos, the distance between the ear and the kidney
was significantly increased in *hdac5* morphants and unchanged in *hdac4* and *hdac6* morphants (Figure 2.2 N, p<.005).

**Inhibition of Class II HDACs Disrupts Primary Cloacal Cilia**

Cilia are present in the zebrafish pronephros in both the ducts and the cloaca. Ductal cilia are approximately 9µm in length and are derived from both mono- and multi-cilia. Cloacal cilia are primary cilia that are shorter at approximately 3µm in length and rotate (Kramer-Zucker et al., 2005) (Sarmah et al., 2007) (Rothschild et al., 2011). In both *pkd2* and *camk2g1* morphants, cloacal cilia is disrupted while ductal cilia is unaffected (Rothschild et al., 2011). In *hdac5* morphants, cloacal cilia is disassembled at 72hpf. Any remaining cloacal cilia in *hdac5* morphants were immotile and much shorter in length. Cloacal cilia were also affected in *hdac4* morphants though in an opposite way. Cloacal cilia were still able to rotate but were elongated to an average length of 5µm in *hdac4* morphants (Figure 2.3 A-F, p<.005). The length of cloacal cilia in *hdac4* morphants could be rescued by a co-injection of the *hdac4* morpholino and 100ng of cherry-*hdac4* mRNA (Figure 2.3 F, p<.005). In order to assure that these defects were not caused by a lack of specification of kidney cells, expression of *cdh17* and *pax2* (two early kidney markers) was assessed and found to be normal in all morphants embryos (data not shown). Interestingly, no defects were seen in cloacal or ductal cilia in *hdac6* morphants (Figure 2.3 A-F).

Primary cilia were also shown to be disrupted in the organ of asymmetry in *pkd2* and *camk2g1* morphants. The primary cilia in the organ of asymmetry, known was the Kupffer’s vesicle (KV), get resorbed when PKD2 or CaMK-II is inhibited (Francescatto et al 2010). In order to assess whether the KV was also affected in class II *hdac* morphants, cardiac asymmetry was examined using *cmic2*. In both *hdac4* and *hdac5* morphants cardiac asymmetry was lost at 24hpf. However, it was unaffected in *hdac6* morphants. This loss of asymmetry in *hdac4* morphants could
be recovered by co-injection of *cherry-hdac4* mRNA and it could be partially recovered in *hdac5* morphants by co-injection of mouse EGFP-HDAC5 (Figure 2.3 G-J). Primary cilia in the KV was similarly disrupted as cloacal cilia in the *hdac* morphants. *Hdac4* morphants showed slightly longer cilia in the KV, while *hdac5* morphants exhibited shorter cilia (Figure 2.3 K-N).

**HDAC Inhibitors Cause Defects in Kidney Development Similar to HDAC Morphants**

TSA is a pan HDAC inhibitor that is known to be effective at inhibiting class I, II, and IV HDACs in zebrafish (Cao et al., 2009b; Bertrand, 2010; de Groh et al., 2010). Zebrafish embryos treated with up to 200nM of TSA were viable and showed no defects in gross morphology. However, both ductal convolution and kidney cell migration were both negatively affected with TSA treatment similar to *hdac5* morphants (Figure 2.4 E,F, p<.005). Embryos treated with TSA also displayed a loss of left-right asymmetry and cloacal cilia were elongated in a similar manner as *hdac4* morphants (Figure 2.4 A-D). Embryos treated with tubastatin A and tubacin, a HDAC6-specific inhibitor, showed no defects in left-right asymmetry or kidney morphogenesis, again mimicking the phenotype of *hdac6* morphants (Butler et al., 2010) (Figure 2.4 D-F).

**Inhibition of HDAC4 in *camk2g1* Morphants Partially Rescues Ductal Convolution**

Epistatic interactions between CaMK-II and Class II HDACs were evaluated by co-injection of *camk2g1* morpholino and either *hdac4, hdac5*, or *hdac6* to evaluate whether the phenotypic defects seen in *camk2g1* morphants was exacerbated or rescued. Ductal convolution is lost in embryos with inhibited *camk2g1*. However, suppression of *hdac4* in *camk2g1* morphants partially restored ductal convolution. This was also seen with treatment of TSA (Figure 2.5 A-P).
Interestingly, suppression of *hdac5* in *camk2g1* morphants exacerbated the morphants phenotype, causing branching of the pronephric ducts. This branching is similar to what was observed when embryos were injected with a kidney targeted dominant negative CaMK-II (Figure 2.5 A-P) (Rothschild et al, 2011). It is of note that although co-injection of *hdac4* MO and *camk2g1* MO partially rescued ductal convolution, cell migration was not rescued, indicated by an increase in ear-kidney distance when compared to control embryos (Figure 2.5 Q). This suggest separate mechanisms mediate ductal convolution and cell migration during kidney morphogenesis.

**Suppression of HDAC4 in Camk2g1 Morphants Prevents Cilia Disassembly**

Epistatic interaction between *camk2g1* and class II HDACs were further evaluated by assessing the effects of suppressed *hdac4* and *hdac5* on the cloacal cilia of *camk2g1* morphants. Inhibition of *camk2g1* in zebrafish embryos causes disassembly of cloacal cilia (Rothschild et al. 2011). Suppression of *hdac4* in *camk2g1* morphants was able to prevent cilia disassembly in the cloaca, increasing the average number of cloacal cilia from 1 to an average of 8 (Figure 2.6 A-F, p<.005). The cloacal cilia in embryos co-injected with *hdac4* MO and *camk2g1* MO were similar in length to control embryos (3 µm) and fully functional (Figure 2.6 G, p<.005). These results were also mimicked with TSA treatment (Figure 2.6 E-G, p<.005). Co-injection of *hdac5* MO and *camk2g1* MO did not rescue cloacal cilia as the average number present in co-injected embryos was 1 (Figure 2.6 A-F, p<.005). The remaining cilia that were present were shorter in length as previously seen in *camk2g1* morphants (Figure 2.6 G, p<.005).
**Subcellular Localization of HDAC4 is altered with CaMK-II Inhibition**

CaMK-II is known to directly influence the subcellular localization of HDAC4 in muscle tissue as well as influence the localization of HDAC5 in the presence of HDAC4 (Backs et al., 2006). To determine how suppression of CaMK-II influences localization of HDAC4 and HDAC5, EGFP-tagged HDAC4 and HDAC5 constructs were injected with and without the *camk2g1* MO. Muscle cells expressing the EGFP-tagged HDAC4 or HDAC5 show predominantly nuclear expression, which remain unchanged with suppression of *camk2g1* (Figure 2.7 A,B,E,F). This was the same for pronephric cells expressing EGFP-tagged HDAC5, with expression being predominantly nuclear with and without the presence of the *camk2g1* MO (Figure 2.7 C,D). However, localization of HDAC4 in pronephric cells was altered with suppression of *camk2g1*. Suppression of *camk2g1* caused a shift in EGFP-HDAC4 localization from predominately nuclear to cytosolic. This suggests a specific role of CaMK-II to retain HDAC4 in the cytosol during kidney morphogenesis (Figure 2.7 G,H).

**Assessing the Effects of Nuclear and Cytosolic Targeted HDAC4 and HDAC5 Expression Constructs**

In muscle tissue, HDAC4 is CaMK-II responsive due to a specific docking domain and two CaMK-II phosphorylation sites at sites S467 and S632 (sites S458 and S606 in zebrafish). Phosphorylation at these sites causes translocation from the nucleus to the cytosol. HDAC5 is CaMK-II responsive in the presence of HDAC4 through the use of its phosphorylation sites at S259 and S498 (sites S251 and S480 in zebrafish) (Backs et al, 2008). Previous results show that CaMK-II affects localization of HDAC4 in pronephric cells as well. In order to determine whether
phosphorylation at these sites is necessary for correct localization in pronephric cells, four kidney targeted constructs were generated that localize HDAC4 and HDAC5 either to the nuclear or to the cytosol. The nuclear targeted constructs were generated by causing a point mutation at the CaMK-II responsive sites changing the serine to an alanine, thereby inhibiting phosphorylation.

The cytosolic targeting constructs were made by changing the serine to either an aspartic acid or a Glutamic Acid, which mimics phosphorylation at these sites and should localize it to the cytosol. Preliminary studies with these constructs analyzed ductal convolution in injected embryos. This resulted in a loss of convolution in 50% of nuclear targeted HDAC4 injected embryos, while embryos expressing cytosolic HDAC5 remained unaffected (Figure 2.8) (Figure 2.9).
Discussion

This study aimed to investigate the link between calcium signaling and epigenetic regulation by histone deacetylases during kidney morphogenesis. It also provides new insight into potential therapeutic targets for autosomal dominant polycystic kidney disease (ADPKD) patients. Calcium is a necessary signaling component for kidney morphogenesis, with Ca^{2+} flux during development occurring in part through the cation channel encoded for by the gene PKD2, a gene whose mutation is known to cause the development of polycystic kidney disease (Igarishi and Somlo, 2002) (He et al, 2011). In zebrafish models of ADPKD, loss of calcium signaling through mutations in pkd2 cause a decrease in the activity of Ca^{2+}/calmodulin dependent protein kinase 2 (CaMK-II) (Rothschild et al, 2011). Loss of CaMK-II activity causes defects similar to pkd2 morphants including the development of anterior pronephric cysts, loss of cell migration and ductal convolution in the pronephros, as well as defects in left-right asymmetry, hydrocephaly, and dorsal-axis curvature (Rothschild et al, 2011). Although CaMK-II is known to be necessary for kidney morphogenesis, the downstream targets of CaMK-II activity in the pronephros have yet to be elucidated.

Associations between CaMK-II and Class II HDACs have been reported in cardiac tissue (Backs et al, 2008). In cardiac tissue, HDAC4 is directly phosphorylated at sites S467 and S632 by CaMK-II inducing the binding of chaperone proteins and sequestration in the cytosol (Backs et al, 2008). This study is the first to demonstrate that CaMK-II has a similar effect on HDAC4 in pronephric cells during kidney morphogenesis. Suppression of *camk2g1* caused translocation of HDAC4 into the nucleus thereby preventing transcription of its target genes. Autonomous CaMK-II is present in both the anterior and posterior pronephric duct and loss of activity causes specific defects in those regions (Rothschild et al, 2011). Therefore, it is likely that the sequestration of
HDAC4 in the cytosol enables transcription of specific target genes for the anterior and posterior pronephros.

Anterior pronephric defects caused by inhibition of \textit{camk2g1} activity include loss of cellular migration and ductal convolution (Rothschild et al, 2011). CaMK-II has previously been associated with focal adhesions turnover in cell culture. Suppression of CaMK-II activity caused an increase in the size of focal adhesion complexes and a decrease in cell motility (Easley et al, 2008). In contrast, constitutively active CaMK-II caused a loss of phospho-tyrosine levels in focal adhesion kinase (FAK) and paxillin causing a reduction in the size of focal adhesion complexes and a decrease in cell motility (Easley et al, 2008). As cell motility and convolution defects are seen in the pronephric ducts of \textit{camk2g1} morphants and convolution defects are rescued with \textit{hdac4} inhibition, it is possible that targets of \textit{hdac4} include genes that promote focal adhesion turnover, enabling ductal convolution to take place. However, these results also suggest that pronephric cell migration and convolution involves several pathways, as convolution was rescued in \textit{camk2g1} morphants with \textit{hdac4} suppression but the pronephric cells still were unable to migration to their normal anterior position.

Posterior defects in \textit{camk2g1} morphants include loss of cloacal cilia development and stability. Inhibition of \textit{hdac4} caused elongation of cloacal cilia suggesting that targets of \textit{hdac4} promote primary cilia formation in the cloaca. This could occur through transcription of a gene called missing in metastasis (MIM). This gene has been previously identified as being required in the basal bodies for cilia maintenance (Bershteyn et al., 2010). This gene is also known to be a target of MEF2C, which has previously been established to be regulated by HDAC4 (Saarikangas et al, 2011). This suggest that a possible mechanism could be that sequestration of \textit{hdac4} in the
cytosol by *camk2g1* enables *mef2c* gene transcription in the posterior kidney, which in turn promotes cilia formation through transcription of *mim*.

In mouse and zebrafish models, suppression of HDAC through pharmacological inhibition reduced cyst formation in *pkd2* mutant and morphants (Cao et al, 2009) (Xia et al, 2010). Similar results were produced with the use of the pan-HDAC inhibitor TSA, where treatment was able to prevent cyst formation in *camk2g1* morphants as well as rescue ductal convolution, ciliary length, and cell migration. Although our results show that both *hdac4* and *hdac5* play roles during kidney morphogenesis, our results suggest that TSA acts to rescue kidney development primarily though inhibition of *hdac4*.

Previous research has shown that HDAC5 can be responsive to HDAC4 in the presence of CaMK-II (Backs et al, 2008). However, unlike HDAC4, HDAC5 does not possess a specific CaMK-II docking site, and it is only responsive through interactions with HDAC4. Loss of *hdac5* activity resulted in defects in ductal convolution and cell migration as well as caused the development of kidney cysts (Backs et al, 2008). In contrast to *hdac4*, suppression of *hdac5* was not able to rescue ductal convolution or cell migration in *camk2g1* morphants. In fact, defects were exacerbated with branching of the pronephric ducts occurring. This suggest that although both *camk2g1* and *hdac5* influence kidney morphogenesis, they work in separate pathways. This was also supported by *camk2g1* inability to affect the localization of *hdac5*.

Results from this study lead to the following proposed model of action. During normal development, calcium flux though the cation channel PKD2 enables activation of CaMK-II. CaMK-II is then able to become autonomous and interact with HDAC4, retaining it the cytosol allowing for transcription of its target genes. In *camk2g1* inhibited embryos, HDAC4 is unable to be retained in the cytosol, so it translocates to the nucleus, where it inhibits transcription of genes.
necessary for development (Figure 2.10). Future studies will include the use of kidney targeted HDAC4 and HDAC5 constructs that should localize to either the nucleus or the cytosol in order to further validate this model. The inactive HDAC4 and HDAC5 constructs have a mutation preventing phosphorylation known CaMK-II phosphorylation sites. Based upon our model, this mutation should cause HDAC4 to localize to the nucleus as it is unable to be phosphorylated by CaMK-II. The cytosolic targeted HDAC4 contained a phosphomemetic mutation the two known CaMK-II phosphorylation sites which should cause its retention in the cytosol. Based upon previous research, it is expected that the same mutations will cause nuclear or cytosolic localization in the HDAC5 construct as well. However, these two sites are CaMK-II responsive sites. As this data suggest that CaMK-II and HDAC5 are working in separate pathways to affect kidney morphogenesis, it is possible that it is phosphorylation at other sites that affect HDAC5 activity during development.

This study newly identifies HDAC4 as a target of CaMK-II during kidney morphogenesis. It also provides a potential area of interest for new therapeutic targets for ADPKD. However, this study also demonstrates that specific pharmacological inhibitors may be necessary when designing treatments for ADPKD. Although inhibition of hdac4 was able to rescue convolution in camk2g1 morphants, those defects were exacerbated by inhibition of hdac5. Based on these results, investigators should aim for more specific methods of treatment as these two proteins appear to have opposing effects on kidney development.
Figure 2.1 Morphology of class II hdac morphants: Embryos were injected with 2ng of control, 2ng of hdac4, 2ng of hdac5, or 8ng of hdac6 MO. Gross morphology was assessed at 24hpf, 48hpf, and 72hpf (A-L). Scale bar = 500 µm. HDAC4 morphants showed orofacial defects including partial cleft (N) compared to control embryos (M) at 72hpf. Scale bar = 100µm. Acetylated histone H3 levels were assessed at 48hpf in control embryos (O,Q), 2ng hdac4 MO (P), or 2ng hdac5 MO (R). Scale bar = 5µm. Acetylated α-tubulin was assessed in control (S) and 8ng hdac6 MO (T) embryos. Scale bar = 500µm. Semiquantitative RT-PCR reaction analyzed the expression of class II HDACs during development (U-W). Expression was also assessed using whole-mount in situ hybridization at the one cell stage and 48hpf (X, Y, Z).
Figure 2.2 Kidney cysts are visible in *hdac5* morphants. Dorsal views of 48hpf embryos injected with 2ng control, 2ng *hdac4*, 2ng of *hdac5*, or 8ng of *hdac6* MO (A-D). Kidney cysts are visible in *hdac5* morphants (C). Class II HDAC morphants were immunostained for α1 Na⁺K⁺ATPase at 72hpf and imaged at low magnification (E-H) and high magnification (I-L). Scale bar = 100µm. *Hdac5* morphant embryos show loss of ductal convolution (M) and loss of anterior migration (N). n=40-70 embryos per experiment. *P < 0.005.
Figure 2.3. Inhibition of *hdac4* and *hdac5* cause opposing effects on primary cilia. Lateral views of primary cilia in the junction of a pronephric duct with the cloaca in 72hpf embryos immunostained for acetylated tubulin (A-D). Scale bar = 5 μm. Length and number of cloacal cilia were assessed at 72hpf and compared to controls (E,F). *n* = 15–30 embryos. *P* < 0.005. Cardiac asymmetry was analyzed by *eml2* expression at 24 hpf (G–I) and plotted as a percentage of embryos (J). *n* = 20–38 embryos. Primary cilia length in the KV at the 12-somite stage (K–M) is averaged (N). *n* = 40–60 cilia. Scale bar = 5 μm. *P* < 0.005.
Figure 2.4. Pharmacological inhibition of class II HDACs mimic morphant phenotype. Embryos were treated with 0–200nM trichostatin A or 10μM tubastatin A beginning at the cleavage state. Cloacal cilia were imaged at 72hpf (A,B) and averaged (C). Scale bar = 5μm. n = 15–30 embryos. Cardiac asymmetry was assessed at 30hpf (D). n = 30–40 embryos. Anterior kidney convolution (E) and kidney ear distance (F) were determined at 72hpf. n = 15–26 embryos. *P < 0.005.
Figure 2.5. CaMK-II and HDACs work in the same molecular pathway enabling anterior kidney morphogenesis. Embryos were injected with *camk2g1* MO (1 ng), *hdac4* MO (2 ng), *hdac5* MO (2 ng), *camk2g1* and *hdac* MOs together, or the *camk2g1* MO followed by bud-stage trichostatin A treatment. Embryos were laterally imaged at 72hpf using DIC optics (A–E) or imaged dorsally using immunofluorescence of α1 Na+/K+ATPase (F–O). Scale bars = 100μm. Arrowheads in I and N indicate adventitious lumens, and arrows indicate branched ducts. Convolution was assessed at 72hpf (P) and pronephric migration was assessed by posterior ear-anterior kidney distance in μm at 72hpf (Q). n = 30–41 embryos.
Figure 2.6. CaMK-II and HDACs work in the same molecular pathway leading to cloacal cilia development and stabilization. Embryos were injected with the camk2g1 MO (CaMKII; 2 ng), hdac4 MO (2 ng), hdac5 MO (2 ng), camk2g1 and hdac MOs together, or the camk2g1 MO followed by bud-stage trichostatin A treatment. Embryos were imaged at 72 hpf after immunolabeling with acetylated tubulin (A–E, green). Scale bar = 5 μm. Cilia number and length were assessed at 72 hpf and compared to control embryos and camk2g1 morphants (F,G). *P < 0.005 compared to control embryos. **P < 0.005. N = 25–62 cilia.
Figure 2.7. HDAC4 subcellular localization is altered with inhibition of *camk2g1*. Embryos were injected with EGFP-HDAC5 (30 ng/μl) alone (A,C), EGFP HDAC5 and *camk2g1* MO (1 ng) (B,D), EGFP-HDAC4 (30 ng/μl) alone (E,G), and EGFP-HDAC4 and *camk2g1* MO (1ng) (F,H). Embryos were analyzed for subcellular localization in muscle cells (A,B,E,F) and kidney cells (C,D,G,H) at 24hpf and 48hpf. n = 15–20 embryos. Scale bar = 5μm.
Figure 2.8. Ductal convolution in nuclear targeted HDAC4 embryos. Anterior convolution was assessed in embryos injected at the 1-4 cell stage with 30ng of Cherry-HDAC4 S^{488}A S^{606}A. Convolution was observed by immunostaining the kidney specific NaKATPase. Ductal convolution was varied in HDAC4 S^{488}A S^{606}A embryos with half having ductal convolution (A) and half lacking ductal convolution (B). N=4.
Figure 2.9: Ductal convolution in cytosolic targeted HDAC5 embryos. Anterior convolution was assessed in embryos injected at the 1-2 cell stage with 30ng of EGFP-HDAC5 S^{251}A S^{480}A. Convolution was observed by immunostaining the kidney specific NaKATPase. Convolution was observed in control embryos (A, n=2) as well as in HDAC5 S^{251}A S^{480}A embryos (B, n=2)
Figure 2.10. Model of action. Ca$^{2+}$ flux through PKD2 activates CaMK-II to sequester HDAC4 in the cytosol. Sequestration of HDAC4 in the cytosol reduces allows for relaxation of the chromatin and transcription of HDAC4-target genes. Loss of PKD2 or CaMK-II induced the release of HDAC4 translocate to which then translocated to the nucleus to inhibit target expression.
Chapter 3: Investigations into the Role of CaMK-II in Kidney Cell Migration

Abstract

The serine/threonine protein kinase CaMK-II is known to play several roles during zebrafish kidney development. These include ciliary stability, pronephric migration, and pronephric convolution, although the exact mechanisms of these actions are currently unknown. This study aims to further understand these pathways by investigating three potential mechanisms for CaMK-II's involvement in kidney development. In this study, camk2g1 morphants had decreased rates of kidney cell migration when compared to controls, causing a decrease in the distance traveled by migrating cells. Previous research has shown that loss of CaMK-II activity increases the size of focal adhesion complexes resulting in decreased migratory capabilities. In addition, alterations in expression of the extracellular matrix protein collagen is caused by loss of pkd2, a cation channel necessary for CaMK-II activity. As interactions between migrating cells and the ECM are necessary for focal adhesion formation and turnover, alterations in collagen expression was assessed in camk2g1 morphants as a possible cause for the decreased rate of migration. Suppression of camk2g1 caused increases in col2a1a present in the notochord. Suppression of collagen secretion using the secretory inhibitor Brefeldin A was able to partially rescue pronephric convolution in morphants embryos. The decreased rate of migration in camk2g1 morphants could also be caused by loss of notch signaling. Notch signaling is necessary for cell differentiation in the zebrafish kidney and loss of notch signaling is associated with a loss of cellular migration in drosophila. Inhibition of camk2g1 caused alterations in notch signaling proteins in the cloaca which could be restored with inhibition of HDAC4, indicating that notch signaling proteins may be downstream of CaMK-II and HDAC4 activity during kidney morphogenesis. This study
contributes to the understanding of the various roles of CaMK-II during kidney morphogenesis. Although it is likely that the effects of camk2g1 inhibition seen in this study is the result of several developmental pathways being affected, it is possible that CaMK-II regulation of the subcellular localization of HDAC4 directly or indirectly regulates notch signaling pathways, which in turn affects collagen expression and cellular migration. Further studies will be necessary to understand the potential connections between these pathways.
Introduction

Autosomal dominant polycystic kidney disease is one of the most common heritable disease in the world (Igarishi and Somlo, 2002). It is also a disease that in spite of its prevalence, little treatment options exists. Treatment for this disease currently consist of lifestyle changes such as a healthier diet and exercise, and medication to alleviate the symptom from the kidney’s reduced function, such as medications for blood pressure (Helal, 2015). The lack of treatments for this disease is due partially to gaps in understanding about disease progression. Mutations in genes PKD1 and PKD2 are known to be the primary causes of ADPKD, with reduced calcium signaling occurring due to these mutation (Igarishi and Somlo, 2002).

Research occurring within the past 10 years has identified CaMK-II as a downstream effector of the cation channel PKD2 (Rothschild et al, 2011). In zebrafish models, inhibition of camk2g1, the predominant form of CaMK-II present in the kidney, causes a loss of cell migration, loss of ductal convolution, and destabilization of cloacal cilia. In addition, zebrafish with inhibited camk2g1 also display dorsal axis curvature and develope anterior kidney cyst, mimicking zebrafish pkd2 morphants (Rothschild et al, 2011) (Mangos et al., 2010) (Obara et al., 2006) (Schottenfeld et al., 2007). Histone deacetylase 4 (HDAC4) is also affected by a reduction in camk2g1 activity. Suppression of CaMK-II causes translocation of HDAC4 from its normal location in the cytosol to the nucleus, thereby inhibiting its target genes (Rothschild et al, 2018). Co-inhibition of CaMK-II and HDAC4 is able to recue ductal convolution in camk2g1 morphants, leading to the model in which CaMK-II works to retain HDAC4 in the cytosol allowing for transcription of its target genes, thereby promoting kidney development (Rothschild et al, 2018).

In order to develop effective therapeutic techniques for ADPKD patients, this study aims to further understand how CaMK-II influences kidney development by investigating its roles in
cellular migration during morphogenesis. Inhibition of CaMK-II is known to cause a loss of kidney cell migration, but specifically how suppression of CaMK-II activity affects migration is currently unknown. One possibility, is that CaMK-II affects the rate of cell migration during development. During kidney morphogenesis, specified pronephric cells undergo epithelialization at approximately 18hpf before migrating anteriorly where they undergo ductal convolution in order to connect with the glomerulus (Drummond and Davidson, 2010). Kidney cells maintain specific rates of migration at distinct periods of development. Prior to 24hpf, kidney cells migrate slowly, at a rate of approximately 2µm/hour. Beginning at 28.5hpf and continuing until 3dpf, this rate increases to 6µm/hour (Vasilyev et al, 2009). This increase in rate corresponds to cell proliferation in the distal segment of the pronephric kidney and the start of active fluid transport into the pronephric lumen (Vasilyev et al, 2009). Embryos with downregulated CaMK-II exhibit a dramatic decrease in anterior migration, as measured by the distance between the back of the ear and the top of the kidney, at 72hpf (Rothschild, et al, 2011). Therefore, it is possible that inhibition of CaMK-II is affecting the rate at which specified kidney cells migrate, thereby preventing connection with the glomerulus and kidney function.

If CaMK-II is affecting the rate at which kidney cells migrate during development, then it is important to understand specifically what is slowing migrating cells. During development, kidney cells migrate via collective cell migration. Collective cell migration is reliant upon the formation of focal adhesion complexes that form when the extracellular matrix proteins interact with integrin receptors in motile cells (Easley et al, 2008). Alteration in extracellular matrix components have long been associated with cyst development. Human ADPKD cells exhibit an overproduction of extracellular matrix components when compared to normal cells (Wilson et al, 1992). These cells also have decreased migratory capabilities due to their increased adhesiveness
to the extracellular matrix component collagen (Drummond, 2011). Alterations in ECM proteins have also been seen in animal models of ADPKD. In mouse models, kidney cyst development is associates with irregular expression of several ECM proteins including collagen and laminin (Ebihara et al, 1995).

Zebrafish models for ADPKD exhibit similar alterations in ECM components. Body curvature is a consistent phenotype seen in morphants for both *pkd2* and *camk2g1* (Schottenfeld et al, 2007) (Rothschild et al, 2011). This body curvature is associated with an increased amount of type II collagen present in the notochord sheath (Mangos et al, 2010). Zebrafish *pkd2* morphants also have an increased expression of *col2a1*, *col9a2*, and *col27a1* in the notochord due to alterations in post translational modification (Mangos et al, 2010) (Le Corre et al, 2014). This increase is associated with an increase in COPII secretory proteins, proteins are responsible for collagen trafficking from the ER to the Golgi (Le Corre et al, 2014). Increases in expression of these proteins led to the idea that alterations in ADPKD ECM components could be associated with alterations in secretion of ECM proteins from the notochord to the kidney (Le Corre et al, 2014). In fact, zebrafish *pkd2* morphants treated with Brefeldin A, a pan COPII dependent secretory inhibitor, showed a significant reduction in cyst formation as well as a rescue of dorsal axis structure (Le Corre et al, 2014). As *camk2g1* morphants exhibit the same body curvature as *pkd2* morphants, it is possible that loss of CaMK-II activity causes an increase in collagen expression which increases kidney cells adhesiveness to the ECM during morphogenesis, thereby slowing and/or preventing migration (Rothschild et al, 2011). This would also correspond with previous studies, showing that pharmacological inhibition of CaMK-II causes a decrease in cell motility and an increase in the size of focal adhesion complexes (Easley et al, 2008).
Another pathway that may play a role in cellular migration during kidney development is the notch signaling pathway. Notch signaling acts through receptor-ligand interactions which causes the cleavage of the notch intracellular domain (NICD) (Bray, 2016). The NICD then translocates to the nucleus where, through interactions with other regulatory proteins, it mediates transcription of its target genes (Bray, 2016). This pathway is involved in a number of biological processes including cell fate, stem cell regulation, and heart morphogenesis (Fuss et al, 2004) (Bray, 2016). It also has roles in disease pathologies such as certain cancers and polycystic kidney disease (Bray, 2016) (Idowu et al, 2018).

Notch signaling has previously established roles in kidney development through cell fate determination of transporting epithelia and multi-ciliated cells in the pronephric ducts (Liu et al, 2007). In addition, regulation of notch signaling has been previously linked to both CaMK-II and HDACs. Pharmacological inhibition of CaMK-II caused a loss of Notch-1 signaling in cell culture (Mamaeva et al, 2008) while treatment with TSA, a pan-HDAC inhibitor, reduced Notch-3 protein levels in human leukemia cell lines (Pinazza et al, 2018). Inhibited Notch signaling has also been linked to reduced cell migration in Drosophila (Fuss et al, 2004). Therefore, it is possible that notch signaling components may be the next step in the previously establish pathway of PKD2 activated CaMK-II regulating HDAC4 during kidney development (Rothschild et al, 2018). It is hypothesized that inhibition of CaMK-II will cause a loss of notch signaling, possibly though the alterations of the subcellular localization of HDAC4.
Methods

**Zebrafish Strains and Care**

Zebrafish embryos were obtained through natural matings and raised at 28.5°C (Kimmel et al., 1995). Zebrafish strains used were Wild-type (AB and WIK), Tg(Tp1:GFP), and Tg(NakATPase:GFP).

**Morpholino Injections**

Translation-blocking antisense morpholinos were designed to target zebrafish *camk2g1* (1ng), *hdac4* (2ng), and *hdac5* (2ng), purchased from Gene Tools along with a control mismatch morpholino (2ng). All morpholinos were used as previously described with an injection volume of 1nl (Francescatto et al., 2010) (Rothschild et al., 2011) (Rothschild et al., 2013). The morpholinos for *hdac4* and *hdac5* were designed to target the following the regions around the translation start site: *hdac4*: CGCCACACTCACATCAACCATCAGC; nucleotides -11 to +14, *hdac5*: GATCTTCCCTGCCTGAAGTTCTCCA; nucleotides -38 to -14).

**Whole Mount In Situ Hybridization**

Digoxigenin-labeled antisense riboprobes were synthesized as previously described using T3 or T7 RNA polymerase (Rothschild et al., 2007). Embryos used for whole-mount *in situ* hybridization (WISH) were developed using alkaline phosphatase–conjugated anti-digoxigenin. The col9a2 probe was designed using the following primers: Sense 5’ AGGTGCT
ACCGGAATGATTG 3’, Antisense 5’ GGATCCATTAACCCTCACTAACGGGAGGTCCAG GTCGTCCTG 3’. The col27a1a probe was designed using the following primers: Sense 5’ AACCACAAGGGCAAGAATTG 3’, Antisense 5’ CAGGTGTTGTTGTTGTTGGA 3’. DeltaC and Col2a1a were provided by Wilson Clements, Ph.D. at St. Jude Children’s Research Hospital. Probes for Hdac4 and hdac5 were designed to correspond to sequences amplified using the primer sets previously mentioned.

**Drug Treatment**

Control zebrafish embryos and camk2g1 morphants were incubated in 5 μM Brefeldin A (BFA) in 1.9ml of E3 water with 1.0μl PTU starting at 24hpf continuing until fixing at 48hpf.

**Fluorescent Localization**

Embryos were fixed in 4% paraformaldehye or Dent’s fixative as previously described (Rothschild et al., 2011). Embryos were then incubated with mouse anti-α 1 Na+/K+-ATPase (Developmental Studies Hybridoma Bank, University of Iowa [α6F] at a 1:10 dilution). After overnight incubation in primary antibody, embryos were incubated with the secondary antibody goat anti-rabbit Alexa568 (Invitrogen at 2.5 μg/ml) and imaged using confocal microscopy. Embryos were imaged either on a Nikon E600 compound microscope using a 10X dry, 20X dry, or 40X dry objective, or on Nikon AZ-100 Macro Zoom fluorescent stereo microscope.
**Video Microscopy**

Embryos were imaged by confocal microscopy (Nikon C2 Plus two-laser) on a Nikon Eclipse Ni compound microscope using a 20X dry objective or on a Nikon AZ-100 macro zoom fluorescent stereo microscope using Elements AR 4.50 software. Embryos used for live imaging for kidney migration rates were anesthetized and mounted on a slide using 1.0% low melting agarose (product name). Embryos used for *tp1* expression images were anesthetized and mounted directly only a slide with E3 water.

**CaMK-II Expression Constructs**

Kidney targeted WT and K^{43}A CaMK-II expression constructs using the Na^{+}/K^{+}-ATPase promoter (Liu et al., 2007) were generated using Gateway technology as previously described (Invitrogen) (Francescatto et al., 2010).

**DeltaC Rescue**

*Hdac4* MO (2ng) and *camk2g1* MO (1ng) were co-injected into zebrafish embryos at the one cell stage. *DeltaC* expression was assessed at 24hpf using WISH as described above.

**Statistical Analysis**

Statistical significance was set at P<0.05 using the two-tailed *t*-test
Results

**Pronephric cell migration is unaffected in DN CaMK-II injected embryos**

In order to assess whether inhibition of CaMK-II affects the rate of migration of kidney cells, zebrafish embryos were injected with either a kidney targeted cherry-tagged WT CaMK-II or a kidney targeted GFP-tagged dominant negative (DN) CaMK-II which lacks phosphotransferase ability. This resulted in embryos with mosaic expression, where fluorescent kidney cells expressed the injected construct and non-fluorescent cells remained unaffected. Migration rates were first assessed in WT CaMK-II embryos. Fluorescent cells present in the medial portion of the pronephros moved a total distance of 28.3µm between 50hpf to 55hpf or 5.6µm/hr (Figure 3.1). This is consistent with previous finding stating that pronephric cells migrate faster than their normal 2µm/hr during the time period between 28.5hpf and 3dpf (Vasilyev et al, 2009). WT CaMK-II positive cells in the distal regions of the kidney migrated slower at 2.2µm/hr (data not shown). This is also consistent with previously published data as the distal portion of the pronephros is necessary for cell proliferation and not migration (Vasilyev et al, 2009). This rate of migration was unaffected by the injection of DN CaMK-II. Kidney cells expressing DN CaMK-II had a migrated a total of 22µm from 48hpf to 53hpf, retaining an increased rate of migration of 4.4µm/hr (Figure 3.2) (Supplemental Video 3.1). Embryos expressing DN CaMK-II also exhibited no defects in cell proliferation, as cell division was visible in the distal portion of the pronephros (Supplemental Video 3.2).
**Pronephric cell migration is decreased in camk2g1 morphants**

In order to evaluate the rate of migration of kidney cells in camk2g1 morphants, NakATPase:EGFP embryos were injected with 1ng of camk2g1 morpholino. The rate of migration in uninjected embryos was found to be consistent with previous published data (Vasilyev et al, 2009). Pronephric cells migrated 27.22µm between 49hpf and 50hpf or 5.44µm/hr (Figure 3.3) (Supplemental Video 3.3). Camk2g1 morphants do not reach this increased rate of migration. Pronephric cells in camk2g1 morphants migrated a total of 8.75µm between 54hpf and 59hpf or 1.75µm/hr (Figure 3.4) (Supplemental Video 3.4). This migration rate of camk2g1 morphants is lower than both uninjected embryos and embryos injected with WT CaMK-II. Interestingly, it’s also reduced when compared to embryos injected with DN CaMK-II (Figure 3.5). This could be due to the camk2g1 morpholino being more evenly distributed among pronephric cells compared to the kidney targeted DN CaMK-II embryos.

**Col2a1a expression is increased in camk2g1 morphants**

Dorsal axis curvature is a common phenotype in zebrafish pkd2 morphants as well as camk2g1 morphants (Schottenfeld et al, 2007) (Rothschild et al, 2011). In pkd2 morphants, this body curvature was suggested to be due to an increased amount of col2a1a, col9a2, and col27a1a expression (Mangos et al, 2010). In order to determine if this is also true for camk2g1 morphants, whole mount in situ hybridization was used to assess col2a1a expression at 48hpf in both uninjected embryos and camk2g1 morphants. Similar to pkd2 morphants, col2a1a expression was dramatically increased in the notochord at 48hpf (Figure 3.6). Col9a2 expression was also found to be upregulated in pkd2 morphants. However, it was unchanged in camk2g1 morphants at 48hpf.
(Figure 3.6). Preliminary data suggest that there may be an increased in col27a1a expression in camk2g1 morphants, similar to what was seen in pkd2 morphants (data not shown). However, this requires further examination.

**Treatment with secretory inhibitor Brefeldin A partially rescue ductal convolution in camk2g1 morphants**

Increases in col2a1a levels in pkd2 morphants was found to be related to an increase in complex II Sec proteins pointing to an increase in collagen secretion (Le Corre et al, 2014). Treatment with Brefeldin A (BFA) a COPII secretory inhibitor, not only prevented body curvature in pkd2 morphants, but also reduced cyst formation as well (Le Corre et al, 2014). As camk2g1 morphants also exhibit increases in col2a1a expression, it was of interested to determine whether treatment with BFA could rescue kidney defects seen in camk2g1 morphants, namely the loss of ductal convolution. Treatment with 5µM of BFA did not affect development of uninjected wild type embryos but was able to restore ductal convolution in camk2g1 morphants (Figure 3.7) (Figure 3.8). Convolution was seen in just under 50% of embryos treated with 5µm of BFA, compared to only approximately 20% in untreated camk2g1 morphants (Figure 3.8).

**Notch Signaling is decreased in camk2g1 morphants**

The notch signaling pathway is known to be necessary for kidney morphogenesis (Liu et al, 2007). In addition, it has also been linked to both CaMK-II and HDACs in cell culture (Mamaeva et al, 2008) (Pinazza et al, 2018). In order to determine if inhibition of CaMK-II is altering notch signaling during development, the camk2g1 morpholino was injected into transgenic
embryos expressing GFP labeled \textit{tp1}, a notch response element (Parsons et al, 2009). \textit{Tp1} expression can be seen in the zebrafish cloaca at 24hpf as well as 48hpf (Figure 3.9) However, this expression is reduced or lost in \textit{camk2g1} morphants (Figure 3.9). Uninjected embryos had an average of 3 \textit{tp1} positive cells in the cloaca at 24hpf and 5 \textit{tp1} positive cells at 48hpf. This number was significantly decreased in \textit{camk2g1}, where most embryos have no \textit{tp1} expression at 24hpf and only 2-3 positive cells at 48hpf (Figure 3.10, p<.05).

\textit{DeltaC} expression is increased in the cloaca in \textit{camk2g1} morphants and \textit{hdac5} morphants

In order to further investigate the connections between notch signaling and the CaMK-II-HDAC4 pathways during kidney morphogenesis, expression of the notch ligand \textit{deltaC} was assessed using WISH in embryos injected with the \textit{camk2g1} MO, \textit{hdac4} MO, or the \textit{hdac5} MO. \textit{DeltaC} expression was unchanged in \textit{HDAC4} morphants, but was increased in the cloaca in \textit{hdac5} morphants (Figure 3.11). Inhibition of \textit{camk2g1} also caused an increase in \textit{deltaC} expression in the cloaca at 24hpf (Figure 3.11). This increase in expression was not seen at 48hpf (Figure 3.12). The increase in \textit{deltaC} expression is consistent with previous findings on the regulation of Delta ligands by CaMK-II in the inner ear, where \textit{camk2g1} morphants shows increased levels of \textit{deltaA} and \textit{deltaD} expression (Rothschild et al, 2013). The increase in \textit{deltaC} expression seen in \textit{camk2g1} morphants could indicate a feedback loop in which a loss of response from the receptor could signal for increased ligand production in an effort to complete the signaling pathway.
**Suppression of HDAC4 in *camk2g1* morphants rescues deltaC expression**

Pronephric duct convolution is lost when *camk2g1* activity is inhibited and is able to be rescued with co-suppression of *hdac4*. In order to determine whether *hdac4* is able to have the same effect on the notch signaling pathway, *deltaC* expression was assessed in embryos injected with both the *hdac4* MO and *camkg1* MO. *DeltaC* expression is increased in the cloaca at 24hpf in *camk2g1* morphants. (Figure 3.11). This increased expression is prevented in *camk2g1* morphants with suppressed *hdac4* (Figure 3.13). This suggest a possible role for CaMK-II-HDAC4 pathway in the regulation of notch signaling during kidney morphogenesis. This could occur through direct regulation of *deltaC* expression or through regulation of the Notch receptors, as CaMK-II has previously been shown to regulate Notch signaling in cell culture (Ann et al., 2012) (Mamaeva et al., 2009).
Discussion

This study aimed to investigate the role of CaMK-II in the previously reported migratory defects associated with zebrafish models of ADPKD. Inhibition of camk2gl in zebrafish embryos causes a loss of cell migration in kidney cells (Rothschild et al, 2011). This study has determined that this could be due to an inability of migrating cells to reach an increased rate of migration normally seen between 28.5hpf and 3dpf (Vasilyev et al, 2009).

Formation of the zebrafish pronephros is reliant upon the anterior migration of pronephric cells to a specific location where they can convolute inward and connect with the glomerulus (Vasilyev et al, 2009). This migration occurs collectively, with cells maintaining apical cell-cell junctions. Cell migration is initiated at the start of active fluid transport to the pronephros, after which migrating cells form transient focal adhesion complexes with the basement membrane, while extending lamellipodia in the direction of movement (Vasilyev et al, 2009).

The formation and subsequent disassembly of focal adhesion complexes is an integral step in cell migration as cells use them as anchor points to pull themselves along during migration. Focal adhesion complexes are multi-protein structures that form when integrins in the migrating cell interact with the ECM, inducing autophosphorylation of focal adhesion kinase (FAK), which in turn recruits src, paxillin, and a number of other focal adhesion proteins (Easley et al, 2008). Disassembly of focal adhesion complexes is associated with transient elevations in Ca$^{2+}$ and CaMK-II has been shown to have a role in focal adhesion turnover by inducing the dephosphorylation of FAK and paxillin (Easley et al, 2008).

In cell culture, alterations in CaMK-II activity inhibited cell motility and caused alterations in FAK and paxillin phosphorylation in focal adhesion complexes (Easley et al, 2008).
Pharmacological inhibition of CaMK-II over stabilized paxillin and caused an increase in the size of focal adhesion complexes (Easley et al, 2008). Conversely, constitutively active CaMK-II caused a decrease in FAK and paxillin phosphorylation, resulting in a decrease in cell motility as cells were less adhesive to the ECM (Easley et al, 2008).

In this study, *camk2g1* morphants exhibited a slower rate of migration during the time period in which increased migration rate in essential in order to form a functional kidney. As previous research has indicated that loss of CaMK-II causes over stabilization of focal adhesion complex, it is possible that there is a loss of focal adhesion turnover in *camk2g1* morphants, inhibiting migration. Future research should focus on assessing expression of focal adhesion proteins during kidney morphogenesis, specifically FAK and paxillin as these are known to be responsive to alteration in CaMK-II activity.

It is of note that this decrease in migration was only seen in embryos injected with the *camk2g1* morpholino and not in embryos injected with the DN form of CaMK-II, K43A. This could be due to the more even distribution of the morpholino among kidney cells. In the course of this study, injection of the DN CaMK-II construct produced fluorescence in approximately 10 or less kidney cells per embryos and often these were distributed along the length of the pronephros, occurring primarily in the medial and distal portions. Previously, expression of DN CaMK-II was shown to cause significant decreases in cell migration as well as cause abnormal branching in the pronephros. However, in that instance, positive cell were clustered at the anterior end as opposed to being more evenly distributed as seen in this study (Rothschild et al, 2011). As kidney cells migrate collectively, with each cell maintaining cell junctions while following the path of the cell on the leading end, it is likely that the disruption in migration was caused by the leading edge cells expressing DN CaMK-II (Rothschild et al, 2011). In the current study, the leading edge cells
remained wild type, while the cells expressing DN CaMK-II were medial and distal. Therefore, it is likely that because the cells migrate collectively, the wild type cells were able to compensate for any defects in the GFP positive cells in the middle, preventing any alterations in migration rate.

In addition to alterations in focal adhesion complexes, this study points to another potential pathway where an increase in col2a1a in camk2g1 morphants may be a cause of reduced migration. Collective cell migration is reliant upon formation and disassembly of focal adhesion between migrating cells and the extracellular matrix (ECM) (Vasilyev et al, 2009). As increases in collagen density have been reported to slow or prevent migration in cell culture, increase in collagen expression may be preventing migration during kidney development (Kuzcek et al, 2019). This is further supported by a reduction in kidney defects with treatment of Brefeldin A, a secretory inhibitor previously shown to reduce secretion of col2a1a in pkd2 morphants (Vasilyev et al, 2009). This potential pathway requires further examination, with future studies focusing on expression of kidney specific collagen protein, such as col27a1a, and other secretory proteins in camk2g1 morphants.

This study has also identified CaMK-II regulation of notch signaling as a potential pathway of interest. This is supported by a decrease in tp1 expression in the cloaca at 24hpf and 48hpf. Inhibition of camk2g1 also caused an increase in deltaC expression in the cloaca, suggesting a feedback loop where increased expression of the notch ligand is attempting to compensate for the loss of response from the receptor (Ma and Jiang, 2007). The increase in deltaC was able to be rescued by suppression of hdac4, mimicking previous research where inhibition of hdac4 was able to partially rescue kidney defects in camk2g1 morphants.

It is possible that camk2g1 regulation of notch signaling may play a role in cellular migration. Alterations in notch signaling components were seen in the cloaca, which is the sight
at which inhibition of *camk2g1* was shown to cause a loss of cloacal cilia, potentially inhibiting the fluid flow necessary for migration (Rothschild et al, 2011). In addition, murine cell culture transfected with a constitutively active form of the notch 1 receptor had decreased expression of type II collagen, suggesting that the notch signaling pathway may play a role in collagen regulation (Blaise et al, 2009). Therefore a potential pathway may be that notch activity, regulated by the previously established CaMK-II-HDAC4 pathway, is providing a similar function during kidney morphogenesis, and its inhibition is resulting in increased collagen expression and reduced migration. However, this pathway is speculative, and requires further study for validation.
Figure 3.1. Migration distance in WT CaMK-II embryo. Embryos were injected with 100ng of the WT CaMK-II construct Cherry:CaMK-II. Embryos were imaged every 15 minutes between 50hpf and 55hpf. The imaged cell moved a total of 28.31µm over the five hour time period or 5.66µm/hour.
Figure 3.2. Migration distance in DN CaMK-II embryo. Embryos were injected with 100ng of the DN CaMK-II construct GFP-K$^{43}$A. Embryos were imaged every 15 minutes between 48hpf and 53hpf. The imaged cell moved a total of 22µm over the five hour time period or 4.4µm/hour.
Figure 3.3. Migration distance in *NakATPase:EGFP* embryos. *NakATPase:EGFP* embryos were imaged every 15 minutes between 49hpf and 59hpf. Imaged cells moved an average of 27.22µm over the five hour time period or 5.44 µm/hour.
Figure 3.4. Migration distance in *camk2g1* morphants. *NakATPase:EGFP* embryos were injected with 1 ng of *camk2g1* morpholino. Embryos were imaged every 15 minutes between 54hpf and 59hpf. The imaged cell moved a total of 8.75µm over the five hour time period or 1.75µm/hour.
Figure 3.5. Average migration distance WT and CaMK-II inhibited embryos. Migration rates were analyzed in embryos injected with 100ng of Cherry:CaMK-II, 100ng of DN CaMK-II construct, or 1ng of camk2g1 MO, as well as uninjected NakATPase:EGFP embryos. There was no difference in migration rates between WT CaMK-II (number of positive cells = 3), DN CaMK-II (number of positive cells = 6), or NakATPase:EGFP embryos (number of positive cells = 3). However, preliminary data suggest there may be a decrease in cell migration rates in camk2g1 morphants (number of positive cells = 2).
Figure 3.6. Col2a1a expression is increased at 48hpf in camk2g1 morphants. Col2a1a and Col9a2 expression was assessed at 48hpf using anti-sense RNA probes and WISH. Col2a1a expression is increased in the notochord at 48hpf in camk2g1 morphants (n=9) when compared to control (n=8). Col9a2 is unchanged in camk2g1 morphants (n=7) when compared to control (n=6).
Figure 3.7: Ductal convolution in camk2g1 morphants treated with secretory inhibitor Brefeldin A (BFA). Anterior convolution was assessed at 48hpf in camk2g1 morphants treated with secretory inhibitory, Brefeldin A. Convolution was observed by immunostaining the kidney specific NaKATPase in untreated controls (A, D), untreated camk2g1 morphants (B, E), and camk2g1 morphants treated with 5µM of Brefeldin A (C, F).
Figure 3.8. Ductal convolution in *camk2g1* morphants treated with secretory inhibitor Brefeldin A (BFA). Anterior convolution was assessed at 48hpf in controls (n=19), controls treated with 5µM BFA (n=11), *camk2g1* morphants (n=19), and *camk2g1* morphants treated with 5µM of BFA (n=20).
Figure 3.9. *Tp1* expression in control embryos and *camk2g1* morphants. The number of GFP positive cells in the cloaca was assessed in uninjected *Tp1:GFP* embryos and in *Tp1:GFP* embryos injected with 1ng of *camk2g1* morpholino at 48hpf. *Camk2g1* morphants have a loss of *tp1* expression at 48hpf.
Figure 3.10. Number of *tp1* positive cells in the cloaca in *camk2g1* morphants. The number of GFP positive cells in the cloaca was assessed in uninjected Tp1:GFP embryos and in Tp1:GFP embryos injected with 1ng of *camk2g1* morpholino. Embryos were assessed at 24hpf (A, control n=25, *camk2g1* MO n=25) and again at 48hpf (B, control n=12, *camk2g1* MO n=15).
Figure 3.11. Inhibition of *camk2g1* and *hdac5* causing an increase in *deltaC* expression in the cloaca at 24hpf. Embryos were injected with 1ng of *camk2g1* MO, 2ng of *hdac4* MO, or 2ng *hdac5* MO. *DeltaC* expression was assessed using an anti-sense RNA probe and WISH at 24hpf.
Figure 3.12. *DeltaC* expression is unchanged at 48hpf. Embryos were injected with 1ng of *camk2g1* MO at the 1-4 cell stage. *DeltaC* expression was visualized at 48hpf using an anit-sense RNA probe and WISH. *DeltaC* expression was unchanged in *camk2g1* morphants (n=16) at 48hpf when compared to controls (n=20)
Figure 3.13. *DeltaC* expression is restored to wildtype level in *camk2g1* MO with co-inhibition of *hdac4*. Embryos were co-injected with 1ng of *camk2g1* MO and 2ng *hdac4* MO. *DeltaC* expression was assessed at 24hpf using WISH.
Supplemental Video 3.1. Migration Rate of Kidney Cells in a DN CaMK-II Embryo. Live imaging of a DN CaMK-II injected embryo taken between 48hpf and 53hpf showing pronephric cells migrating at a rate of 4.40μm/hr
Supplemental Video 3.2. Cell Division in a DN CaMK-II Embryo. Live imaging of a DN CaMK-II injected embryo showing cell division in the distal portion of the pronephros.
Supplemental Video 3.3. Migration Rate of Kidney Cells in a NaKATPase:EGFP Embryo. Live imaging of a NaKATPase:EGFP embryo taken between 50hpf and 55hpf showing pronephric cells migrating at a rate of 5.44µm/hr.
Supplemental Video 3.4. Migration Rate of Kidney Cells in *camk2g1* Morphant Embryo.
Live imaging of a *camk2g1* morphant embryo taken between 54hpf and 59hpf showing pronephric cells migrating at a rate of 1.75μm/hr.
Chapter 4: Genetic compensation in *camk2g1* mutants

**Abstract**

CaMK-II is a Ca\(^{2+}\)/calmodulin depending protein kinase encoded by 4 genes (α, β, γ, δ) in humans, and 7 genes (α, β1, β2, γ1, γ2, δ1, δ2) in zebrafish. Since being first described in the 1970s, CaMK-II has been found in virtually every tissue type and is involved in a variety of cellular processes including long term potentiation, ion channel regulation, and organogenesis. In zebrafish embryos with inhibited *camk2g1*, a variety of developmental defects can be seen including defects in kidney morphogenesis, the development of pronephric kidney cysts, left-right asymmetry defects, hydrocephaly, ciliary instability, and aural defects. In an effort to further understand the roles of *camk2g1* during development, a homozygous mutant was generated in zebrafish using TALEN technology. However, the mutant exhibited none of the phenotypic defects seen in zebrafish *camk2g1* morphants. qPCR analysis of CaMK-II expression in *camk2g1* mutant embryos showed a 3.5 fold increase in *camk2g2*, its paralog. In addition, several of the other CaMK-II genes showed a decrease in expression up to 40%, suggesting genetic cross-talk during development. Generation of G0 *camk2g1* mutants using CRISPR/Cas9 exhibited nearly all of the defects seen in *camk2g1* morphants, including kidney defects, cyst development, and ciliary defects. These results validate the *camk2g1* morphant phenotype seen in current and previous studies, and indicate that genetic compensation does not occur in morphants or G0 mutants, but is developed over time in stable mutant lines. This study demonstrates the ability of CaMK-II genes to genetically compensate for the loss of their paralog and validates the use of knockdown methods such as morpholinos for future developmental studies.
**Introduction**

**TALEN and CRISPR/Cas9 Genome Editing**

Transcription activator-like effectors (TALEs) are proteins secreted by bacteria that contain a DNA binding domain enabling them to effect gene transcription in vivo (Nemudryi et al, 2014). The DNA binding domain of TALEs proteins is formed by monomers made from 34 amino acids with a variable region at positions 12 and 13 (Moscue and Bogdanove, 2009). These positions follow a specific code enabling targeting to one of the four nucleotides based upon which two amino acids are present in the variable region. The amino acid in position 12 is necessary for stabilization and the amino acid in position 13 directly interacts with the targeted nucleotide (Moscue and Bogdanove, 2009).

Once this system was understood, an artificial DNA binding domain was created and placed in a construct with the catalytic domain of FokI, a restriction endonuclease, yielding the transcription activator-like effector nucleases (TALEN) genome editing method (Nemudryi et al, 2014). This method allows for the use of TALEs monomers designed to target specific regions of DNA. TALENs are used in pairs and are designed to sit on opposite strands of the targeted sequence (Nemudryi et al, 2014). The FokI domains on the C-terminus end of the TALENs dimerize with their counterpart on the opposite strand, causing a double stranding break. This break is then repaired by nonhomologous end joining (NHEJ) an error prone process that often causes insertions or deletions disrupting the function of the target gene (Lieber, 2010) (Nemudryi et al, 2014).

Another reliable, commonly used method of genome editing is the CRISPR/Cas9 system. This method is also derived from bacteria (Nemudryi et al, 2014). However, in contrast to the
TALEN system, CRISPR/Cas9 relies upon a complex consisting of non-coding RNA and the Cas9 endonuclease. In bacterial cells, a small sequence of invading DNA is inserted into the CRISPR locus (Nemundryi et al, 2014). This sequences is complementary to the sequence on the foreign DNA which is located near a specific region known as the PAM (protospacer adjacent motif) sequence necessary for the targeting of the CRISPR/Cas9 complex (Mojica et al, 2009). After insertion of the fragment of foreign DNA into the CRISPR locus, it is transcribed, allowing for targeting of the CRISPR/Cas9 complex to the invading DNA, enabling its degradation (Nemudryi et al, 2014).

The use of CRISPR/Cas9 in research settings has become a common and reliable method of genome editing. In mice and zebrafish, the typical method used is to create a single guide RNA which consist of a designed crRNA targeting the area of interest in the genome and the tracrRNA which enables the activity of the CRISPR/Cas9 complex. This is microinjected along with either Cas9 protein or mRNA (Chang et al, 2013) (Wang et al, 2013). Similar to the TALEN system, once the CRISPR/Cas9 complex binds to its target site, a double stranded break occurs. This break in then repaired by the error prone NHEJ, leading to inhibition of activity of the target gene (Nemudryi et al, 2014).

Although the CRISPR/Cas9 system has proven to be a reliable method for gene editing, some genes have proven resistant to targeted mutations (Gagnon et al, 2014). In order for the CRISPR/Cas9 system to be effective, a double stranded break must generate a repair that causes a frameshift mutation and/or a premature stop codon (Nemudryi et al, 2014). In 2014, researchers attempted to use CRISPR/Cas9 to generate a zebrafish camk2g1 mutant. Microinjection of sgRNA and Cas9 mRNA was able to successfully generate alteration in the camk2g1 gene in 8 injected zebrafish embryos. However, none of these insertion/deletions generated a frameshift, leaving a
fully function protein, suggesting biological repair mechanisms designed to reduce allele diversity (Gagnon et al, 2014).

**Genetic Compensation**

The use of genome editing methods like TALEN and CRISPR/Cas9 have become the standard in reverse genomic studies. However, research has shown discrepancies between the phenotypes of stable mutants and morphants for particular genes. In fact, a study comparing the phenotypes of mutants and morphants for 24 specific genes found that only 5 of the compared genes shared a similar phenotype (Kok et al, 2015). This was further demonstrated through their own study where 10 of the developed and validated zebrafish mutant lines showed none of the developmental defects previously published in morphants for the same genes. This led researchers to the conclusion that many morphant phenotypes are due to off target effects of the morpholino and are not reliable descriptions of gene function (Kok et al, 2015).

The discrepancy between mutant and morphants phenotypes have been described in a variety of model organism including zebrafish, mice, and human cell lines (Kok et al, 2015) (Daude et al, 2012) (Karakas et al, 2007). Although this was originally thought to be due to off target effects of knockdown mechanisms, recent studies have indicated that genetic compensation in mutant models may be preventing any developmental defects to due genetic loss (El-Brolosy and Stainier, 2017). A comparison between a zebrafish mutant and morphant for egf17, an extracellular matrix gene, showed opposing phenotypes, with egf17 mutants developing normally and egf17 morphants having severe vascular defects. Addition analysis found that other extracellular matrix genes were upregulated in egf17 mutants, not in morphants indicating
compensation by these genes (Rossi et al, 2015). Other studies have produced similar results, where zebrafish mutants for epoa, a red blood cell regulator, have increased levels of epob expression, preventing developmental defects (She et al, 2019).

Genetic compensation has been well reported in a variety of studies using a number of model organisms. However, the exact mechanisms of genetic compensation are currently unknown. Based upon previously reported studies, several possible explanations exists for the induction of genetic compensation in mutant models. One thought is that DNA damage causes epigenetic changes that induce genetic compensation (El-Brolosy and Stainier, 2017). When DNA is damaged in some way, chromatin remodelers and histone-modifying enzymes act to de-condense and reorganize chromatin (El-Brolosy and Stainier, 2017). It has been proposed that when a mutation occurs, these same mechanism may de-condense chromatin around compensating genes inducing their upregulation. This idea has been supported by studies in both Drosophila and C elegans (Stukenholz et al, 1999) (Raj et al, 2010).

Another proposed method for genetic compensation is that compensation is induced by the presence of mutant mRNA (El-Brolosy and Stainier, 2017). The presence of mutant mRNA induces the nonsense mediated decay (NMD) pathway (Schuermann, et al, 2015). In zebrafish, two different mutations were made in the same exon for the gene mt2 which caused two different levels of NMD activation. The mutant with the higher level on NMD had lesser phenotypic defects that the mutant with lower levels of NMD response (Schuermann et al, 2015). Several potential pathways have been proposed for how the presence of mutant mRNA can induce genetic compensation. One potential pathway is that mutant mRNA acts to bring transcription factors and chromatin remodelers to the site of compensation genes (El-Brolosy and Stainier, 2017). It has also been proposed that mutant mRNA could also act through RNA binding proteins (RBPs). RBPs
act to stabilize mRNA and if the mutant mRNA is unable to bind to the RBPs then they would become available to stabilize the compensating genes (El Broslosy and Stainier, 2017).

In this study, a zebrafish mutant for the gene $camk2g1$ was analyzed for the presence of genetic compensation. Previous research has shown that inhibition of $camk2g1$ using morpholinos and dominant negative constructs yielded embryos with severe phenotypic defects including hydrocephaly, left-right asymmetry defects, kidney defects, and body curvature (Francescatto et al, 2010) (Rothschild et al, 2011) (Rothschild et al, 2018). The $camk2g1$ mutant possesses none of these defects and develops normally. This study aims to validate previously published works on $camk2g1$ using knockdown mechanisms while also investigating genetic compensation by other CaMK-II family members in $camk2g1$ mutants.
Methods

Zebrafish strains and care
Wild type (AB and WIK) fish embryos were obtained through natural matings and raised at 28.5°C as previously described (Kimmel et al., 1995).

Generation of camk2g1 mutant zebrafish and sequence analysis
Camk2g1 mutants were generated and provided by the Thisselab using the following protocol. The TALEN to target camk2g1 was designed using TAL Effector Nucleotide Targeter 2.0 (https://tale-nt.cac.cornell.edu/node/add/talen-old), assembled by using the Golden Gate TALEN and TAL Effector Kit 2.0 (Cermak et al., 2011) and cloned into the pCS2+TAL3DD or pCS2+TAL3RR (Dahlem et al., 2012). The sequence in the exon 2: TTGCAGGGGTGCTTCTCggggtgcggagatgtgTGAAAAATCCACTGGCCA has been targeted using the TALEN modules= NG NN HD NI NN NN NN NG NN HD HD NG NG HD NG HD and NN NN HD HD NI NN NG NN NN NI NG NG NG NG NG NG HD NI. Synthesized capped mRNAs were injected into eggs of the AB/Tu strain. Characterization of mutant alleles in F1 adults was assessed by performing an endonuclease assay using AcI that cuts at GCGG (underlined in the sequence above) in the linker sequence. The primers used to amplify the DNA fragment around the target site were 5’- TGCATGCCTATAAGGTGCATTGT-3’ and 5’-GCAACCACACACACAGACACCTCG-3’. PCR amplification of genomic DNA with these primers (Meeker et al., 2007) results in production of a 329 bp fragment that is cut into fragments of 88 bp and 241 bp by the AcI endonuclease. AcI resistant fragments potentially carrying mutations were cloned into pCR2.1 TOPO and sequenced. F1 frame shift mutations predicted to truncate the translated camk2g1 protein were crossed with each other to generate F2 homozygous mutant embryos.
Crosses between *camk2g1* heterozygous adults identified at each generation with the genotyping method described above were used to maintain the mutant strain population. Mutations were confirmed by PCR amplification of *camk2g1* exon2 followed by sequencing.

**Measurement of CaMKII activity**

Control, *camk2g1* mutants, and embryos injected with the *camk2g1* translation blocking morpholino (MO) were dechorionated and lysed as previously described. Total CaMKII activity was assessed by measuring phosphate incorporation into autocamtide2, as previously described (Rothschild et al., 2007).

**Morpholino injections**

Translation-blocking antisense morpholino oligonucleotide (MO) were used to target zebrafish *camk2g1* and *camk2g2*. The *camk2g1* MO (1ng or 0.5ng) and the *camk2g2* MO (10ng) were previously described and validated (Francescatto et al., 2010; Rothschild et al., 2011; Rothschild et al., 2013).

**Immunofluorescence and Microscopy**

Fluorescent and transmitted light micrographs were acquired using a Nikon AZ100 macro zoom fluorescent stereo microscope. For fluorescent localization, embryos were fixed in 4% formaldehyde/PBS or Dent’s fixative as previously described (Rothschild et al., 2011). Fixed embryos were incubated with mouse anti-acetylated α-tubulin (Sigma Chemical Co. (T7451) at 2
µg/ml) or mouse anti-α1 Na+/K+-ATPase (Developmental Studies Hybridoma Bank, University of Iowa (α6F) at a 1:10 dilution). Primary antibody incubations were overnight and were followed by either goat anti-mouse Alexa488 or goat anti-rabbit Alexa568 (Invitrogen at 2.5 µg/ml). Embryos were imaged by confocal microscopy (Nikon C2 Plus two-laser) on a Nikon Eclipse Ni compound microscope using a 20X dry objective or on a Nikon AZ-100 macro zoom fluorescent stereo microscope using Elements AR 4.50 software.

**Quantitative PCR**

Embryos were collected from control and camk2g1 mutant embryos at 24 hours post fertilization (hpf), dechorionated, and total RNA was extracted as previously described (Rothschild et al., 2009; Rothschild et al., 2007). Concentration and purity of total RNA was assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). cDNA was prepared as previously described using approximately 1µg of RNA per reaction (Rothschild et al., 2009; Rothschild et al., 2007). qPCR primers were designed against all seven transcriptionally active CaMKII genes (Table 4.1). All reactions were performed in triplicate using 2x SensiFAST SYBR No-ROX Mix (Bioline, BIO-98005). qPCR analysis was performed using a micPCR system (Bio Molecular Systems, M0000636). Single product amplification was confirmed using both a melt curve and direct sequencing of the product. Gene expression fold change was analyzed using the 2^ΔΔCT method and was normalized to elongation-factor 1-alpha (ef1α) (Silver et al., 2006).
RT-PCR and Sequencing

Total RNA from control and camk2g1 mutant embryos at 24hpf was prepared as described above. PCR primers were designed to amplify the variable domain and were used as previously described (Rothschild et al., 2007). PCR products were cloned into the pSC-A vector (Agilent) and sequenced to identify CaMKII gene specific splice variants.

Camk2g1 CRISPR/Cas9 G0 embryos

Four guide RNAs (gRNAs) against camk2g1 were designed using the Dharmacon CRISPR design tool. Two were designed to target the 5’UTR (CCCGTGACTCTGCTGATTGC, ATACTACCATGCTAGCTTTG), one was designed to target exon 1 (TGTAACCTCGACCAGGTTTTA), and one was designed to target exon 2 (GGGGTGCTTTCTCCGTGTTG). The two gRNAs targeting the 5’UTR region were ordered from the Synthego Corporation using their CRISPRevolution sgRNA EZ Kit. The gRNAs targeting exon 1 and exon 2 were designed with the addition of a T7 promoter region (TAATACGACTCACTATA) as well as a constant overlap region (GTTTTAGAGCTAGAAATAGC). Promega GoTaq Hot Start Green Master Mix (M5122) with gRNA primers (GCGTAATACGACTCAGCCACCACCGACTCGGTGCCAC), and constant region oligonucleotides (AAAAGCACCAGACTCGGTGCCACTTTTTCTAAGTTGATAACCGGACTAGCCTTTATTTTAACTTGGCTATTTCTACTCTAAAAC) were used to generate the gRNA templates using the following thermocycler conditions: 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 20 seconds. The final template was purified using the GENECEAN II Kit (MP Biomedicals 1001400). The transcription reaction was performed using the MEGAshortscript T7 High Yield Transcription Kit (AM1354) with a
final purification using the RNA Clean & Concentrator Kit (Zymo Research R1015). The four gRNAs were coinjected at 200ng/μl each with Cas9 protein (1ng) (New England Biosciences) into one-cell stage zebrafish embryos. Insertion/deletions were verified by PCR amplification and sequencing. Primers for camk2g1 5’ UTR (1) (CACTCACTCACACACAAAAACAC, CATTIT AACAAATGGCGAGAG), camk2g1 5’ UTR (2) (GCCACTCTCGCCATTTAAAAATG, CGGGAGGAGAATCTCATGTGGTCC), camk2g1 exon1 (CTCCGTTCCTTCTCTCTCTGGAC, TACAATGTTGCAGCAGCCTTAC).

**Video Microscopy**

Live embryo cilia videos were acquired at 72hpf under differential interference contrast optics using a Nikon 60X water immersion Plan APO objective and 20 frames per second acquisitions after transient anesthesia and immobilization in low melting point agarose, as previously described (Rothschild et al., 2011).

**Statistical methods**

Statistical analysis used the two-tailed t-test and significance was set at P<0.05.
Results

Camk2g1 mutants show no gross morphological defects

Camk2g1 mutant embryos were generated by the Thissse lab using TALEN technology which induced a pre-mature stop codon in exon 2. This stop codon occurred in the catalytic domain by the addition of 8 nucleotides and the loss of 6, producing an inactive kinase (Figure 4.1 A). The mutants are homozygous viable through 8 generations. Analysis of gross morphology show no morphological defects in the camk2g1 mutants when compared to controls (Figure 4.1 B-G). This is in sharp contrast to previous studies inhibiting camk2g1 activity through the use of morpholinos, dominant negative, and pharmacological inhibition (Francescatto et al, 2010) (Rothschild et al, 2011) (Rothschild et al, 2013) (Rothschild et al, 2018). Studies using the previously stated methods resulted in embryos that exhibited defects that include body curvature, hydrocephaly, left-right asymmetry defects, and kidney abnormalities. Analysis of CaMK-II activity showed 20-30% decrease in total CaMK-II activity in camk2g1 mutants when compared to controls, demonstrating successful loss of function in mutant embryos (Figure 4.1 H)

Camk2g1 mutants show no morphological defects when injected with camk2g1 morpholino

The loss of camk2g1 in homozygous mutants causes no morphological defects, in contrast to previous studies using morpholinos to suppress activity (Francescatto et al, 2010) (Rothschild et al, 2011) (Rothschild et al, 2013) (Rothschild et al, 2018). In an effort to further validate the specificity of the camk2g1 morpholino, camk2g1 mutant embryos were injected with 1ng of the camk2g1 morpholino and morphology was compared to controls, un-injected mutant embryos, and camk2g1 morphants. As previously reported, camk2g1 mutants produce no morphological defects
when compared to control embryos (Figure 4.2 A-C, G-I) Camk2g1 morphants exhibit dorsal-axis curvature, hydrocephaly, and defects in cloacal morphology (Figure 4.2 D-F). The injection of 1ng of camk2g1 morpholino into camk2g1 mutant embryos showed none of the defects seen in morphants embryos, with no body curvature present, normal cloaca formation, and no visible brain defects (Figure 4.2, J-L). Analysis of percent inhibition of CaMK-II activity in camk2g1 mutants confirm a loss of CaMK-II activity by almost 30%. However, embryos injected with .5ng or 1ng of camk2g1 morpholino show a greater loss than the camk2g1 mutants. Embryos injected with .5ng have a 40% loss of CaMK-II activity while embryos injected with 1ng of camk2g1 morpholino have a 60% loss (Figure 4.2 M).

**Camk2g1 mutants show no kidney defects when injected with camk2g1 morpholino**

Inhibition of camk2g1 caused severe defects in kidney development in zebrafish embryos that include loss of cell migration, loss ductal convolution, cloacal cilia loss, and the development of pronephric cysts (Rothschild et al, 2011) (Rothschild et al, 2018). Kidney development was evaluated in camk2g1 morphants, camk2g1 mutants, and camk2g1 mutants injected with 1ng of camk2g1 morpholino. Consistent with previous finding, camk2g1 morphants pronephros failed to convolute and had a significant increase in ear-kidney distance indicating a loss of cellular migration (Figure 4.3 A-B, E-F, p<.05). Camk2g1 mutant embryos had normal pronephric development when compared to controls, with over 90% of the embryos having convolution of the pronephric ducts and an ear-kidney distance equivalent to controls (Figure 4.3 C, E-F, p<.05). Camk2g1 mutants injected with the camk2g1 morpholino also had normal kidney development, with convolution in over 90% of embryos and normal kidney cell migration (Figure 4.3 D, E-F, p<.05) (n=20-35 per condition)
Cloacal cilia formation and stability was also assessed in camk2g1 morphants, camk2g1 mutants, and camk2g1 mutants injected with 1ng of camk2g1 morpholino. Previous studies have shown that embryos with suppressed camk2g1 lose cloacal cilia formation and stability and have cloacal occlusions (Rothschild et al, 2011) (Rothschild et al 2018). Camk2g1 morphants mimicked previous studies, with a reduction in the number of cloacal cilia from an average of 12 to 2 as well as significant decrease in the length of remaining cilia (Figure 4.3 G-H, p<.05). Camk2g1 mutants exhibited no changes in cloacal cilia number or length when compared to controls. This was also true for camk2g1 mutants injected with the camk2g1 morpholino (Figure 4.3 G-H, p<.05). These results are consistent with a total loss of camk2g1 in mutant embryos as well as demonstrates the specificity of the morpholino. (n=20 embryos per condition and n=20-50 cilia per condition)

**Genetic Compensation in Camk2g1 Mutants**

In an effort to understand the discrepancy between the phenotypes of camk2g1 mutants and morphants, expression of all 7 CaMK-II genes were assessed using qPCR to look for evidence of genetic compensation. Validated primer sets were used to assess expression of the 7 CaMK-II genes in zebrafish at 24hpf in camk2g1 mutants and control embryos. Results showed a 40-90% decrease in transcript level in camk2a, camk2b1, camk2b2, camk2d1 and camk2d2 while transcript levels of camk2g2 were increase over 3 fold in camk2g1 mutants (Figure 4.4 A). This increase in expression of camk2g2 persists at 48hpf as did the decrease in camk2a, camk2b2 and camk2d1 expression (Figure 4.5) This increase in camk2g2 expression was further validated by amplifying and cloning the variable domains of CaMK-II genes in control and camk2g1 mutant embryos. Sequencing of obtained clones showed an increase in the presence of clones expressing camk2g2 compared to controls. In addition, clones obtained were both nuclear and cytosolic targeted in both
control and mutant embryos, with two new camk2g2 splice variants being identified in clones obtained from camk2g1 mutants. This not only supports the hypothesis of genetic compensation by camk2g2 in camk2g1 mutants but also provides a potential role of camk2g1 regulation of other isoforms (Figure 4.4 B-D).

In order to validate the role of camk2g2 compensation in camk2g1 mutants, a morpholino was used to suppress activity of camk2g2 in wild type embryos and camk2g1 mutants. Embryos injected with 10ng of the camk2g2 morpholino show no gross morphological defects and had no defects in left-right asymmetry when compared to controls. However, when camk2g1 mutants were injected with the camk2g2 morpholino, there was a loss of left-right asymmetry in the majority of injected embryos (Figure 4.4 E). In addition, wild type embryos injected with the camk2g2 morpholino showed no defects in ductal convolution, while camk2g1 mutant embryos injected with the camk2g2 morpholino showed a loss of convolution in over 90% of injected embryos. Cloacal cilia and ear development were not affected by the injection of the camk2g2 morpholino in either wild type embryos or camk2g1 mutants (Figure 4.4 F). These results demonstrate the new-found roles of camk2g2 in camk2g1 deficient embryos.

Camk2g1 CRISPR Mutants Phenocopy Camk2g1 Morphants

As camk2g1 mutants develop normally, CRISPR/Cas9 technology was used in an attempt to study camk2g1 roles in development. Four non-overlapping guide RNAs were designed to target camk2g1. Two were designed to target the 5’UTR. One was designed to target exon1 and the last was designed to target exon2 (Figure 4.6 A). The efficacy of the four designed gRNA were confirmed by sequencing (Figure 4.7). Embryos injected with the 4 guide RNAs exhibited a variety
of phenotypes as would be expected from mosaic expression. However, the majority of injected embryos exhibited developmental defects that mimicked \textit{camk2g1} morphants. These include tail curvatures, brain defects, and smaller otoliths (Figure 4.6 B-G). In addition, kidney cyst were visible in approximately 50\% of injected embryos by 48hpf (Figure 4.6 E).

Expression of all 7 CaMK-II zebrafish genes were assessed in embryos injected with the 4 guide RNAs targeting \textit{camk2g1} using qPCR. The same validated primer set was used as previously described (Table 4.1). Analysis of CaMK-II expression in CRISPR mutants shows a statistically significant decrease in the presence of both \textit{camk2b1} and \textit{camk2g1} and a statistically significant increase in \textit{camk2a} expression (Figure 4.6 H) In contrast to \textit{camk2g1} mutants, there was no statistically significant difference in expression of \textit{camk2g2} in CRISPR mutants (Figure 4.6 H). This supports the hypothesis that it is the increase in \textit{camk2g2} expression in \textit{camk2g1} mutants that is able to compensation for the absence of its paralog gene, and that this compensation is not occurring in embryos mosaically expressing the 4 CRISPR guide RNAs.

\textbf{\textit{Camk2g1} CRISPR mutants exhibit kidney defects that phenocopy \textit{Camk2g1} morphants}

In an effort to further understand the development of \textit{camk2g1} CRISPR mutants, kidney development was assessed by observing ductal convolution, cell migration, and cloacal defects commonly seen in \textit{camk2g1} morphants (Rothschild et al, 2011) (Rothschild et al, 2018). Approximately 80\% of \textit{camk2g1} CRISPR mutants pronephric ducts failed to convolute, with branching occurring in a subset of embryos (Figure 4.8 A-D). Kidney cell migration was also inhibited in \textit{camk2g1} CRISPR mutants, demonstrated by the statistically significant increase in ear-kidney distance in mutants when compared to control (Figure 4.8 E, p<.05). Cloacal cilia were
also affected in mutants, with an average decrease in cloacal cilia present from approximately 11 cilia in controls (Supplemental Video 4.1) to 5 cilia in mutants, with some possessing no cloacal cilia at all (Supplemental video 4.2) (Figure 4.8 F-I, p<.05). Although the number of cloacal cilia present was significantly reduced in camk2g1 CRISPR mutants, the length of remaining cilia were unchanged when compared to controls (Figure 4.8 J). This is likely due to the mosaic expression in camk2g1 CRISPR mutants. Ductal cilia were unaffected in camk2g1 CRISPR mutants when compared to controls (Supplemental Video 4.3 and 4.4). The kidney defects seen in CRISPR mutants phenocopies that of camk2g1 morphants, further supporting the specificity of the morpholino as well as validating the morphants phenotype seen in current and previous studies.
Discussion

CaMK-II is a serine/threonine protein kinase encoded by 4 genes in humans (α, β, γ, δ) and 7 in zebrafish (α, β1, β2, γ1, γ2, δ1, δ2). (Tombes et al, 2003) (Rothschild et al, 2009) It is present in virtually all tissue types and is known to be involved in variety of cellular functions, from long term potentiation to ion channel regulation (Hudman and Schulman, 2002). Through the use of knockdown mechanisms such as morpholinos, dominant negatives, and pharmacological inhibition, roles of CaMK-II have been expanded to include kidney morphogenesis, left-right asymmetry, and inner ear development (Francescatto et al, 2010) (Rothschild et al, 2011) (Rothschild et al, 2018). Further studies into the roles of CaMK-II would benefit from the development of a stable mutant line in zebrafish. However, this has proven difficult, with some CaMK-II genes, namely camk2g1, proving to be resistant to mutations. (Gagnon et al, 2014).

In this study, a camk2g1 mutant was generated in zebrafish using TALEN genomic editing. Although the use of TALENs was successful in generating a premature stop codon in exon 2 of camk2g1, embryos developed normally, with no sign of the defects seen using the previously mentioned knockdown mechanisms. This study was able to identify genetic compensation as a potential cause for the lack of phenotype in these embryos through the upregulation of a paralog gene. It also identified a potential new role for camk2g1 as a regulator for other CaMK-II genes expression during development. In addition, this study was also able to validate previous finding on the role of CaMK-II though validation of the previously used camk2g1 morpholino.

Camk2g1 mutants possess an over three fold increase of the camk2g1 paralog camk2g2. Previous publications have described the evolutionary retention of these genetic paralogs and redundant (El-Brolosy and Stainier, 2017). However, this terminology implies an almost
unnecessary role for these paralogs. The ability of ‘redundant’ genes to compensate for the loss of their paralog provides a compelling argument for their evolutionary retention. In the current study, the increased expression of \textit{camk2g2} in \textit{camk2g1} mutants seems to prevent the severe phenotypic defects seen in morphants, giving a more defined purpose to its evolutionary retention.

Genetic compensation has also been termed “transcriptional adaptation” (El-Brolosy and Stainier, 2017). Exactly how the loss of one gene induces compensation of its paralog or a similar gene is currently unknown but several pathways have been suggested that include a variety of potential signaling partners including chromatin remodelers, transcription factors, and RBPs (El-Brolosy and Stainier, 2017). Deciphering exactly how transcriptional adaptation occurs requires further study. However, genetic knockdown methods are known to not induce genetic compensation, it would seem that the presence of mutant mRNA is necessary to induce transcriptional adaption either through direct contact with its paralog, or though other epigenetic mechanisms.

In addition for providing evidence of genetic compensation in \textit{camk2g1} mutants, this study also supports the use of genetic knockdown methods in developmental studies. Currently, knockout methods such as TALEN and CRISPR/Cas9 are almost required parts of any developmental biology study. However, there have been a large number of phenotypic discrepancies between mutants and morphants in mice, zebrafish, and cell culture (Kok et al, 2015) (Daude et al, 2012) (Karakas et al, 2007). This had led to a distrust in results derived from studies using knockdown methods such as morpholinos. It had been suggested that mutants provide a more accurate depiction of the roles of a gene of interest and that many of the phenotypes derived from knockdown methods are produced from off target effects.
This study in combination with other published works provides evidence that for some genes, morpholinos may provide a more accurate phenotype than their mutant counterparts. Research have shown that some genes, like *camk2g1*, can be resistant to mutations (Gagnon et al, 2014). In addition, the upregulation of *camk2g2* in *camk2g1* mutants seems to prevent the development of the expected morphant phenotype (Rothschild et al, 2011) (Rothschild et al, 2018). This upregulation of paralogs or other genetic family members has been seen in other studies where mutants and morphants did not possess the same phenotype (Rossi et al, 2015) (She et al, 2019). Therefore, it is likely that genetic compensation can account for many of the differences seen between morphant and mutants for genes of interest. If this is the case, then embryos that do not exhibit compensation, i.e. morphants, may provide a more accurate picture for the roles of a particular gene than the mutant in some cases.
Figure 4.1. Characterization of *camk2g1* mutant zebrafish. Electropherograms of exon2 of *camk2g1* in wild type and *camk2g1* “TALEN” mutant embryos show a loss of 8 nts and gain of 6 nts leading to a premature stop codon (A). Camk2g1 mutants appear phenotypically normal at 22s (B, C), 48hpf (D, E) and 4dpf (F, G). CaMKII specific activity (nmoles/min/mg) is reduced by 25-30% at 48hpf and 72hpf in camk2g1 mutants when compared to controls (H).
Figure 4.2. *Camk2g1* mutants injected with *camk2g1* MO develop normally. Control, *camk2g1* morphants, *camk2g1* mutants, and *camk2g1* mutants injected with 1ng of the *camk2g1* MO were imaged at 48hpf (A-L). *Camk2g1* morphants display curved tails (B) smaller otoliths (F) and defects in cloacal morphology (J). Control embryos, *camk2g1* mutants, and camk2g1 mutants injected with the camk2g1 MO do not display such morphological defects. CaMKII activity assays of 72hpf embryos reveal twice the reduction in CaMKII activity in *camk2g1* morphants injected with 0.5ng or 1.0ng of *camk2g1* MO, when compared to *camk2g1* mutants (M).
Figure 4.3. Normal kidney development in *camk2g1* mutants injected with the *camk2g1* MO. Anterior convolution was assessed at 72hpf in control, *camk2g1* morphants, *camk2g1* mutants, and *camk2g1* mutants injected with *camk2g1* morpholino by immunostaining the kidney specific NaKATPase (A-D). Quantification of convolution in the anterior pronephric duct (E). Analysis of cellular migration at 72hpf in the pronephric duct by measuring the distance between the posterior ear and anterior kidney (F). Cloacal cilia number (G) and length (H) were assayed at 72hpf.
Figure 4.4. Molecular and Functional Compensation of camk2g1 mutants by camk2g2. qPCR of CaMK-II genes in camk2g1 mutants at 24hpf relative to ef1α (A). Percentage of clones attributed to each CaMK-II gene after amplification and sequencing of variable domains in control and camk2g1 mutants at 24hpf (n=15-18) as previously described (B) (Rothschild et al., 2007). CaMK-II splice variant exon utilization falls into specific categories (C) (Rothschild et al., 2007). CaMK-II splice variants denoted by gene and categories that were observed in control and camk2g1 TALEN mutant embryos. Previously unreported splice variants of camk2g2 are colored red (D). Cardiac asymmetry was evaluated at 24hpf by live embryo observation under indicated conditions (E). Convolution was assessed by examining immunostained embryos using the kidney specific NaKATPase (F).
Figure 4.5. Relative mRNA Fold Change at 48hpf in camk2g1 Mutants. Relative fold change of CaMK-II genes in camk2g1 mutants relative to ef1α. Camk2g2 shows a statistically significant increase in expression in camk2g1 mutants. Camk2b2, camk2d1, and camk2a show a statistically significant decrease in expression in camk2g1 mutants.
Figure 4.6. Morphological Defects in $camk2g1$ CRISPR Embryos. 4 gRNAs were designed against $camk2g1$ to generate G0 $camk2g1$ CRISPR embryos (A). G0 $camk2g1$ CRISPR embryos show variable phenotypes with shortened anterior posterior axis and tail curvatures evident at 48hpf (C) compared to control embryos (B). Kidney cysts develop by 48hpf (E, asterisks) and were absent in control embryos (D). Otoliths were smaller in $camk2g1$ CRISPR embryos when compared to controls (F, G). Dorsal, anterior to the left (D, E). Lateral, anterior to the left (F, G). qPCR of CaMK-II genes in $camk2g1$ CRISPR embryos at 24hpf relative to ef1α.
Figure 4.7. Genomic confirmation of *camk2g1* CRISPR embryos. Twenty pooled embryos were sequenced using the sense primer for each of the four gRNA target sequences. Electropherograms for control and *camk2g1* CRISPR embryos confirmed insertion/deletions of each gRNA target.
Figure 4.8. Kidney defects in camk2g1 CRISPR embryos. Anterior convolution is reduced in camk2g1 CRISPR embryos compared to controls at 72hpf (A-D). The ear-kidney distance is increased in camk2g1 CRISPR embryos at 72hpf (E). Cloacal cilia number is reduced in camk2g1 CRISPR embryos (F-I). However, length of remaining cloacal cilia in camk2g1 CRISPR embryos is unchained when compared to control embryos (J).
Supplemental Video 4.1. Control embryos have beating cloacal cilia at 72hpf. Shown at 20 frames per second.
Supplemental Video 4.2. *Camk2g1* CRISPR embryos lack beating cilia at 72hpf. Shown at 20 frames per second.
Supplemental Video 4.3. Control embryos have beating ductal cilia at 72hpf. Shown at 20 frames per second.
Supplemental Video 4.4. *Camk2g1* CRISPR embryos have ductal cilia beating at 72hpf. Shown at 20 frames per second.
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<td>GCAACAGCAAAGTGGCTTCCATC</td>
</tr>
<tr>
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**Table 4.1 qPCR Primers.** Primers targeted for the 7 CaMK-II in zebrafish for amplification in qPCR. All primers were designed to target the 3’UTR except camk2b1 and camk2b2 which were designed to target the association domain.
Chapter 5: Reduction of \textit{camk2g1} Activity in Zebrafish Reveal Novel Roles of CaMK-II in Kidney Morphogenesis that may be Imperceptible in Mutant Models Due to Genetic Compensation.

Since their initial use as a model organism in the 1970s, zebrafish have emerged as an essential tool for developmental studies and disease modeling. Their quick developmental time, high rate of gene conservation with humans, and relatively low husbandry cost have enabled zebrafish to be used in a variety of different research fields. For this study, which focuses on kidney development and ADPKD modeling, their transparency during development has enabled real time imaging of pronephric development and the identification of potential therapeutic targets for ADPKD patients.

Previous research identified CaMK-II, a serine/threonine protein kinase, as a downstream target of Ca$^{2+}$ flux though the cation channel formed by PKD2, one the genes most commonly mutated in ADPKD patients. Once activated, CaMK-II acts to regulate a number of pronephric developmental processes including cell migration, ductal convolution, and cloacal cilia development and stability. Inhibition of \textit{camk2g1}, the predominant form of CaMK-II in the zebrafish kidney, not only disrupts the previously mentioned processes, but also causes the development of anterior kidney cysts. The addition of a constitutively active form of CaMK-II was able to inhibit cyst development, as well as prevent other pronephric defects seen in \textit{camk2g1} morphants.

This study expands upon the previously established role of CaMK-II in pronephric development by identifying its regulation of histone deacetylase 4 (HDAC4) as necessary for kidney morphogenesis. Histone deacetylases are known regulators of gene transcription whose presence in the nucleus causes the condensing of chromatin, preventing transcription of its target
genes. In study, *hdac4* was localized predominantly in the cytosol during pronephric development. The suppression of *camk2g1* caused the subcellular translocation of *hdac4* from the cytosol to the nucleus, thereby preventing transcription of genes normally active during organogenesis. Co-inhibition of *hdac4* was able to rescue ductal convolution defects seen in *camk2g1* morphants, identifying histone deacetylases as a potential new target for treatments for ADPKD. However, caution should be used as suppression of *hdac5* exacerbated defects in *camk2g1* morphants.

Further studies into this pathway should focus on identifying other downstream targets. Potential targets identified by this study include notch signaling proteins. Inhibition of *camk2g1* caused the loss of the notch response element *tp1* in the cloaca and the increase in expression of the notch ligand *deltaC*. Similar to earlier studies, the expression of *deltaC* could be rescued in *camk2g1* morphants with suppression of *hdac4* suggesting that notch signaling proteins may be downstream members of that pathway, and a potential target for treatments for ADPKD. Future studies should focus on understanding the connections between these pathways by analysis of other notch signaling proteins in *camk2g1* morphants, such as *notch3* and *jagged2* in order to identify specifically what notch proteins are being affected.

In addition to identifying the regulation of HDAC4 by CaMK-II, this study also further demonstrates the multifactorial roles of CaMK-II during kidney development. Inhibition of *hdac4* was able to rescue ductal convolution in *camk2g1* morphants, but it was unable to rescue cell migration. This suggests that the processes of ductal convolution and cell migration are separate and that CaMK-II plays an integral role in both pathways. Pronephric cells migrate at a slower rate in *camk2g1* morphants when compared to controls, never reaching the site where they should convolute inward to connect with the glomerulus. In an effort to understand this reduction in
cellular migration rate, this study has identified two potential causes for this defect. One is the increase in expression of the extracellular matrix protein collagen. In *camk2g1* morphant, expression of the ECM protein *col2a1a* was increased, mimicking the expression previously seen in *pkd2* morphants. Increase in collagen expression may be a potential cause for the decreased migration as increased collagen expression is associated with reduced migratory capabilities, caused by an increase in focal adhesion attraction to the ECM. This is also supported by the partial rescue of convolution seen in *camk2g1* morphants treated with the secretory inhibitor, Brefeldin A (BFA). Future research into this area would benefit from the analysis of kidney specific collagen protein as well as more specific mean of inhibiting collagen secretion as BFA inhibits all COP II dependent secretory pathways and is likely inhibiting other necessary pathways.

This research, as well as previous studies, has relied heavily upon the use of knockdown mechanisms such as morpholinos, dominant negatives, and pharmacological inhibitors. Currently, the generation of genetic mutants has become a new standard for developmental research. A mutant for *camk2g1* was generated in zebrafish using TALENs. However, the mutants displayed none of the defects seen in previously studies using knockdown methods. This occurrence is not unique to this study, as there have been many other previously reported discrepancies between mutants and morphants in various model organisms. In this case, genetic compensation seems to be the cause of the lack of phenotype in *camk2g1* mutants. qPCR analysis of the 7 CaMK-II genes present in zebrafish identified a 3 fold increase in the expression of the *camk2g1* paralog, *camk2g2* in mutants when compared to controls, preventing any defects caused by the loss of *camk2g1*. This study also demonstrated that compensation by a paralog gene is not an immediate occurrence, as G0 *camk2g1* CRISPR embryos did not display the same increase in *camk2g2* expression and possessed nearly all of the morphological defects seen previously in *camk2g1* morphants. These
results support the findings of previous studies using knockdown mechanisms such as morpholinos, as genetic compensation may occur in mutant models, inhibiting any reverse genetic studies. Future studies should focus on using the compensatory ability of CaMK-II paralogs to determine how and when compensation in initiated. Generational studies with stable lines generated with the 4 gRNAs for camk2g1 could be used in conjunction with qPCR analysis to determine if compensation by camk2g2 is initiated with the first stable generation or if it is developed over several generations.
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Vita

Sarah Rebecca Ingram was born on December 29, 1990 in Petersburg, Virginia. She was raised in Chesterfield Country and homeschooled by her mother through the 10th grade. She then attended and graduated from Westminster Academy in Richmond, Virginia. She earned an Associate of Arts and Sciences from John Tyler Community College in Chester, VA in 2011. She then attended Virginia Commonwealth University and earned a Bachelor of Science in Forensic Science in 2015. She began graduate school in Integrative Life Sciences in 2015 under the guidance of Robert M. Tombes, Ph.D., and Sarah C. Rothschild, Ph.D. During her studies she was a teaching assistant for five semesters of Molecular Biology Capstone Lab and was awarded Outstanding Biology Graduate Teaching Assistant in 2018.

Manuscripts resulting from the present dissertation research
