An Approach to Evaluate Inhibition of Cyclophilin D-Sensitive Mitochondrial Permeability Transition Pore Formation in Chronic Kidney Disease

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An Approach to Evaluate Inhibition of Cyclophilin D-Sensitive Mitochondrial Permeability Transition Pore Formation in Chronic Kidney Disease

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A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

The mitochondrial permeability transition pore (mPTP) contributes to pathological outcomes in various contexts of tissue injury and disease involving mitochondrial perturbation, including in the kidneys. The prominent stressors leading to formation of the mPTP include increased cellular calcium influx and increased reactive oxygen species (ROS) production or accumulation. Subsequent opening of the mPTP can be a key contributor to poor outcome through several possible mechanisms including a decreased ability of cells to produce ATP and the induction of necrotic and apoptotic cell death. Here, we prevented expression of a critical modulator of the mPTP, namely Cyclophilin D (CypD), in mice that develop chronic kidney disease (CKD) by crossing mice with CypD deficiency to those carrying the Col4a3⁻/⁻ mutation modeling Alport nephropathy. The mouse model of Alport nephropathy exhibits increased ROS production and tubule epithelium cell death in the kidney, suggesting that mPTP opening may be occurring and driving disease progression. In the present study, we found that human and murine kidneys with CKD exhibited alterations indicative of mitochondrial dysfunction possibly associated with mPTP formation. CypD deletion, however, did not improve survival or renal function in Alport mice nor did it reduce histopathology. Together, these results demonstrate that inhibition of CypD activity as an approach to limit mPTP formation does not appear to be a viable pharmacologic target for the treatment of Alport nephropathy.
Author’s Biographical Sketch

The author is a 2012 graduate of Westminster College (Salt Lake City, UT), having earned a Bachelor of Science degree in biology. Allie was also presented with an Honors Certificate upon completion of the college’s interdisciplinary Honors Program curriculum. In 2011, the author was awarded the Honors Independent Summer Research Grant for work on isolating hydrocarbon-degrading extremely halophilic archaea from a contaminated hypersaline environment (Great Salt Lake). Since graduating, Allie has performed research relating chronic kidney disease to mitochondrial dysfunction both at the University of Washington (Seattle, WA) and at Biogen (Cambridge, MA). At the time of publication, the author was employed at Novo Nordisk (Seattle, WA) working to advance the next stage of therapeutics for diabetic kidney disease.
Dedication

To R.T.M.
Acknowledgements

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Chapter I.
Introduction

In 2015, an estimated 50,000 individuals died due to renal failure, making kidney disease the ninth leading cause of death in the United States (National Center for Health Statistics (U.S.), 2017). Presently, 1 in 3 Americans are at risk for developing kidney disease, and over 30 million already have chronic kidney disease (CKD), though many remain undiagnosed due to the disease’s early lack of symptoms (National Kidney Foundation, 2017a). CKD often progresses to total renal failure, and patients are required to endure regular dialysis or obtain a kidney transplant to survive. Those with CKD who do not succumb to renal failure, however, instead suffer an increased risk of recurrent myocardial infarction, heart failure, and sudden cardiac death (Herzog et al., 2011). As the US spends a total of $103 billion annually caring for individuals on Medicare with CKD (National Kidney Foundation, 2017b), this disease represents a substantial toll on the country’s economic and human resources.

Current treatments for those with CKD are limited and mainly focus on treating the underlying causes, including reducing blood pressure and limiting over-activity in the immune response (Satou & Gonzalez-Villalobos, 2012). However, these options insufficiently address the varied etiologies of the disease, and the level of care offered to CKD patients is frequently suboptimal (Kausz & Levey, 2002). Novel therapies are thus required to better address this critically unmet need, and it was the goal of this work to investigate a target for drug therapy to ameliorate CKD.
Functional Anatomy of the Kidney

The kidneys are a pair of organs located in the abdomen of all vertebrate organisms. Their main role is to filter the blood, thereby removing wastes, balancing fluid levels, and regulating electrolytes in the bloodstream. The filtrate is then transferred from the kidney, through the ureters, and into the bladder where it is excreted from the body in the form of urine (Figure 1A).

Figure 1. Anatomical Overview of the Kidney. (A) The human urinary system, wherein the kidneys are connected to the bladder via the ureter. (B) Schematic of a bisected kidney demonstrating the relation of the renal cortex to the medulla. (C) Orientation of a nephron relative to the renal cortex and medulla within the kidney. (D) Detailed structure of a nephron. From “Chapter 25: Control of Body Temperature and Water Balance” by N. Campbell, J. Reece, M. Taylor, E. Simon, and J. Dickey, 2009, Biology: Concepts & Connections, Sixth Edition. Copyright © 2009 by Pearson Education, Inc.
The functional, filtering unit of the kidney is called the nephron (Figure 1C), which is composed of the glomerulus, Bowman’s capsule, renal tubules, and the loop of Henle (Figure 1D). Blood flows through glomerular capillaries (Figure 1D, Black arrows), where fluids from the blood are filtered through small slits between a surrounding layer of cells called podocytes, which normally prevent larger molecules such as proteins and red blood cells from leaking through. The filtrate (Figure 1D, Green Arrows) then moves through the proximal convoluted tubule\(^1\) and into the loop of Henle\(^2\). The loop of Henle acts to regulate the concentration of water and salts by either releasing these molecules back into the kidney medulla (Figure 1C) to be reabsorbed by the kidney capillaries or by retaining them within itself before transferring the final solution into the distal convoluted tubule\(^3\). From there, the filtrate transfers into the collection duct for transport out of the kidney.

Beyond the nephron, other important players in kidney health are the endothelial cells that form the organ’s blood vessels and capillaries, which are critical structures for allowing the transport of oxygen and nutrients necessary for normal cellular metabolism. Additionally, the pericytes, which are fibroblast-like cells embedded within the capillary basement membrane (CBM), are also important as they act to stabilize the peritubular endothelial capillary network (Figure 2). Without pericyte support, the endothelial network of vessels becomes fragile and capillary density is lost. These individual parts, when working in sync, thus play a critical role in maintaining vertebrate health.
Etiology and Pathophysiology of Chronic Kidney Disease

Typical causes for CKD include high blood pressure and diabetes, though there are familial forms of the disease as well, as is seen in Alport Syndrome. Unlike other causes of CKD that usually stem from lifestyle and/or environmental factors, patients suffering from Alport nephropathy have genetic mutations that affect the type IV collagen family of proteins (Hudson, Tryggvason, Sundaramoorthy, & Neilson, 2003). These mutations result in disturbed capillary basement membrane formation, which causes a disruption in the glomerular filtration barrier (Heidet & Gubler, 2009). Despite these differing etiologies, however, all CKD patients present similarly by histology: scarring of the glomeruli (glomerulosclerosis), tubule injury (as quantified by loss of brush border), tubular necrosis, and leukocyte infiltration (Deltas, Pierides, & Voskarides, 2013). This thus suggests that there may be a common underlying mechanism that ultimately culminates in CKD.

When characterizing the disease by microscopy, CKD patients present with deposition of pathological extracellular matrix (ECM) in the kidney interstitial space, between the tubules and the peritubular capillaries (Figure 3) (Fligny & Duffield, 2013).
Myofibroblasts are the cells responsible for the deposition of this pathological ECM that becomes scar tissue. The presence of this scar tissue leads to the progressive loss and contraction of the normal kidney architecture, finally ending in kidney atrophy and thus kidney failure.

The origin of kidney myofibroblasts has been a hotly contested issue over the last few decades, with one side arguing that myofibroblasts are the product of tubules undergoing epithelial to mesenchymal transition (EMT) (Zeisberg & Kalluri, 2004), while others argue they derive from pericytes that detach from the CBM and migrate into the interstitial space to transform into myofibroblasts. Mounting evidence, however, indicates that most myofibroblasts originate from pericytes and are not the product of EMT (Figure 3) (Lin, Kisseleva, Brenner, & Duffield, 2008). It is thus currently believed that injury of the tubular epithelium, which results in “cellular crosstalk,” is responsible for activating pericyte-myofibroblast transition, thereby initiating the pro-fibrotic process (Figure 3) (Fligny & Duffield, 2013). Importantly, the proximal tubules are thought to be the main producer of fibrogenic mediators that activate the pericyte-myofibroblast transition (Wu et al., 2013). Thus prevention of stress in and injury to proximal tubule epithelial cells represents an interesting target to decrease or delay induction of the fibrotic process in human patients.

Mitochondrial Dysfunction in Renal Disease Pathogenesis

In healthy tissue, the mitochondria act as the “power houses” of the cell, producing much of the cell’s adenosine triphosphate (ATP) through oxidative
Figure 3. Responses of Injury to the Kidney Tubulo-interstitial Space. Injured epithelial cells signal to pericytes, resulting in detachment from the capillary wall and migration into the interstitium. There the cells become activated and transition into myofibroblasts, where they proliferate and begin depositing ECM leading to interstitial fibrosis. This thus perpetuates epithelial injury, causing dedifferentiation and a thickening of the tubule basement membrane. From "Activation of Pericytes: Recent Insights into Kidney Fibrosis and Microvascular Rarefaction" by C. Fligny and J.S. Duffield, 2013, Current Opinion in Rheumatology, 25(1), p. 84. Copyright © 2013 by Lippincott Williams & Wilkins.

phosphorylation (OxPhos) via the electron transport chain (ETC) (Figure 4). Many cell types, such as the renal proximal tubules, rely heavily on OxPhos to produce the ATP necessary to perform their function. Under normal conditions, the electron donors NADH and FADH\textsubscript{2} feed electrons into Complex I and Complex II, respectively, of the ETC. As the electrons travel through the ETC, some of their energy is released to pump hydrogen ions from the mitochondrial matrix and into the intermembrane space, thereby generating an electrochemical gradient. This membrane potential then allows for the proton-motive force to be used by Complex V to pump protons back into the mitochondrial matrix, coupling OxPhos with ATP synthesis. Under certain situations of stress, however, a cell’s ability to conduct OxPhos is reduced, thus forcing the cell to rely instead on glycolysis to make up for some of the ATP production lost. Importantly, the proximal tubule epithelial cells of the kidney have a low capacity to perform glycolysis (Feldkamp et al., 2009),
thus making this an especially sensitive population for cell stress caused by ATP deprivation.

**Figure 4. Schematic of the Mitochondrial Electron Transport Chain at Steady State.** Under normal conditions, the electron donors NADH and FADH$_2$ feed electrons (e$^-$) into Complex I and Complex II, respectively, of the ETC. As the electrons travel through the ETC, some of their energy is released to pump hydrogen ions (H$^+$) from the mitochondrial matrix and into the intermembrane space, thereby generating an electrochemical gradient. This proton-motive force is then used by Complex V to pump protons back into the mitochondrial matrix, coupling oxidative phosphorylation with ATP synthesis. Modified from “Agilent Seahorse XF Cell Mito Stress Test Kit User Guide” by Agilent Technologies, Incorporated, 2017. Copyright © 2017 by Agilent Technologies, Inc.

Beyond production of ATP, mitochondria act as one of the main producers of reactive oxygen species (ROS). Although most mitochondrial oxygen consumption is efficiently coupled to the production of ATP, a small percentage of oxygen is reduced by electrons within the ETC to produce superoxide (O$_2^-$), which serves as the precursor for most other ROS (Baughman & Mootha, 2006), such as hydrogen peroxide (Figure 5).

During homeostasis, these ROS either act as second messengers or are quickly converted into benign molecules by ROS scavenging enzymes.

In conditions of cell stress, impaired mitochondrial function can result in increased electron leak from the ETC, leading to higher superoxide production. Interestingly, high levels of mitochondrial ROS within the cell have been shown to
decrease ATP production. The mechanism for this response to high levels of ROS is thought to lie in the cells’ activation of uncoupling proteins (UCPs) within the inner membrane of mitochondria (Granger & Kviets, 2015). Once activated, UCPs open and allow hydrogen ions to travel down the proton gradient without producing ATP, thereby uncoupling OxPhos from energy production (Figure 5). As an increasingly high mitochondrial membrane potential has been shown to lead to higher ROS production (Figure 5 inset), this activity by the UCPs functions to keep ROS within the nontoxic range by decreasing the membrane potential. The negative side effect of this activity, however, is that cells which rely metabolically on ATP produced by OxPhos, such as the renal proximal tubular epithelium, will be metabolically affected to a disproportionate level, potentially activating cell stress signaling pathways and cell death in these populations.

In addition to lowering ATP levels, high levels of ROS cause damage to mitochondrial DNA (mtDNA) and other cellular components. Mitochondria are the only organelles within the cell, besides the nucleus, that contain their own DNA and their own machinery for synthesizing RNA and proteins from this mtDNA. Due to the lack of association of mtDNA with protective histones, its close proximity to the inner mitochondrial membrane where ROS are endogenously generated by the ETC, and the high lipid content of mitochondrial membranes that is potentially susceptible to lipid peroxidation chain reactions leading to the formation of hydroperoxides and aldehydes
Figure 5. ROS Production by the Electron Transport Chain and Mitochondrial Uncoupling. It is possible for wayward electrons from the ETC to reduce oxygen within the mitochondria, thereby forming ROS. These ROS then act on uncoupling proteins (UCP) within the mitochondrial inner membrane, allowing for hydrogen ions to follow the gradient from the inter-membrane space into the matrix without being coupled to production of ATP. Thus, the production of ROS is directly related to the level of membrane potential and thus ATP production. From "Reperfusion injury and reactive oxygen species: The evolution of a concept" by D.N. Granger and P.R. Kviety, 2015, *Redox Biology*, 6, p. 533. Copyright © 2015 The Authors. Published by Elsevier B.V.

(Hruszkewycz, 1992), the mtDNA is thought to be particularly susceptible to ROS attack associated with oxidative stress. Continued mtDNA damage ultimately leads to mutations in the mitochondrial genome. As the mtDNA encodes for several proteins that function in complexes I, III, and IV of the ETC (Sherratt, 1991), mutations in these genes could cause further alterations in the flow of electrons through these components of the ETC (Hsieh, Hou, Hsu, & Wei, 1994), leading to electron leak and inefficient ATP production by the ETC. Additionally, complexes I and III are also thought to be the major sites of superoxide production and other ROS (Murphy, 2009). Consequently, mtDNA damage causing defective function in complexes I and III could potentially result in further
superoxide production. Thus, cell stress can initiate a positive feedback loop of mitochondrial dysfunction, characterized by increased ROS production and decreased ATP production, which could induce or further aggravate the condition of the cell.

In human patients and models of CKD, mitochondrial dysfunction has been associated with the pathogenesis of disease. In studies looking at biomarkers of oxidative stress present in the plasma of human CKD patients, evidence was found to suggest that oxidative stress increased as CKD progressed (Oberg et al., 2004). In the Col4a3−/− mouse, an animal model of Alport nephropathy, there is a significant increase in ROS present in the kidney tissue and urine at the peak of clinical disease as measured by renal function (9 weeks of age in the reported model). Additionally, these mice also show a significant increase in renal tubule cell apoptosis and a significant decrease in the RNA and protein levels of Mpv17L (Gomez et al., 2015), a mitochondrial antioxidant restricted to the proximal tubule in the kidney (Chau et al., 2012). In this strain, the earliest histological signs of disease arise at 5 weeks, demonstrating rare segmental sclerosis of the glomeruli. However, as early as 2.5 weeks of age there is a significant increase in whole kidney expression of microRNA-21 (miR21), which is a small noncoding RNA thought to be involved in silencing metabolic pathways critical for ATP generation and ROS regulation (Gomez et al., 2015). This thus suggests that detrimental metabolic changes within the kidney precede histological signs of disease. In a different mouse model of focal segmental glomerulosclerosis (FSGS), researchers demonstrated the presence of elevated production of ROS in the kidney and abnormal mitochondria morphology, also preceding the appearance of clinical disease (Kawakami et al., 2015).
Together these data suggest a role for mitochondrial oxidative stress in CKD development and progression.

**Mitochondrial Permeability Transition Pore Formation In Response to Stress**

In many states of cell stress, including those leading to elevated levels of intracellular calcium (Ca$^{2+}$) or ROS, a pore approximately 3nm in diameter known as the mitochondrial permeability transition pore (mPTP) forms within the mitochondria, linking the inner mitochondrial matrix to the cell cytosol (Figure 6) (Devalaraja-Narashimha, Singaravelu, & Padanilam, 2005; Martin, Lewington, Hammerman, & Padanilam, 2000; Zheng, Devalaraja-Narashimha, Singaravelu, & Padanilam, 2005). Because of this pore formation, proteins and molecules less than 1.5 kDa can freely enter or leave the mitochondrial matrix (Gunter & Pfeiffer, 1990; Halestrap, 2010; Lemasters et al., 1998; Rasola, Sciacovelli, Pantic, & Bernardi, 2010). The physiological role of the mPTP is unknown. However, as ROS production has been shown to be potential-dependent under normal physiological conditions (Granger & Kviety, 2015), it has been hypothesized that mPTP formation may play a part in the regulation of ROS levels. It has been shown in vitro that under physiological conditions at low intracellular calcium levels, opening of the mPTP causes moderate mitochondrial depolarization that results in decreased ROS production (Akopova et al., 2011). Thus, much like the UCPs discussed above, one potential role for mPTP under steady state may lie in attenuating oxidative damage by diminishing ROS formation in the mitochondria, thereby acting as an endogenous mechanism of mild uncoupling of the ETC (Akopova, Nosar, Man'kovskaja, & Sagach, 2013).
Under conditions of extreme cell stress, however, widespread mPTP opening has been shown to cause the mitochondria to swell and even rupture, thereby releasing apoptotic effectors such as cytochrome c from the mitochondrial intermembrane space, resulting in the activation of caspases leading to apoptotic cell death (Basso et al., 2005; Bernardi & Forte, 2007; Doran & Halestrap, 2000; Forte & Bernardi, 2005; Halestrap, 2006; Halestrap, Clarke, & Khaliulin, 2007; Leung & Halestrap, 2008; Martinou & Green, 2001). Furthermore, due to this sudden increase in mitochondrial permeability, the mitochondrial membrane potential is lost and OxPhos becomes fully uncoupled, resulting in a loss of ATP synthesis (Bernardi, Scorrano, Colonna, Petronilli, & Di Lisa, 1999; Crompton, 2000; Halestrap, 2006). As mitochondria are the main producers of ATP within most cells, mPTP formation often leads to energy depletion and necrotic cell death. Moreover, as the renal proximal tubules have a low capacity for glycolysis (Feldkamp et al., 2009), this portion of the nephron is especially susceptible to cell stress and necrosis following mPTP activation. This phenomenon has been noted both histologically (Feldkamp, Kribben, & Weinberg, 2005) and when quantifying ATP in the kidney tissue of mice that had undergone ischemia reperfusion injury (IRI) (Devalarajana-Narashimha, Diener, & Padanilam, 2009).

Targeting Cyclophilin D to Inhibit mPTP Formation in Acute and Chronic Injury

Though the identities of the proteins that compose the mPTP are yet unproven (Baines et al., 2005; Bernardi et al., 1999; Halestrap, 2010; He & Lemasters, 2002), Cyclophilin D (CypD) has been shown to act as a critical modulator for controlling the pore’s opening (Figure 7) (Woodfield, Ruck, Brdiczka, & Halestrap, 1998). CypD activity in mPTP formation was initially supported using Cyclosporine A (CsA), which
**Figure 6. Hypothetical Model of mPTP Activity.** Various cellular stressors, including oxidative stress, cause opening of the mPTP. This allows for free transfer of solutes between the cytosol and the mitochondrial matrix, thereby causing swelling of the mitochondria, the loss of membrane potential, and thus the loss of ATP production. From “Therapeutic Targets” by Congenia, R&D, 2009.

was a known inhibitor of mPTP formation. The mechanism by which CsA functions is through binding to and inhibiting the activity of a subset of peptidylprolyl isomarase (PPIase) immunophilins, described as the cyclophilin superfamily based on their binding affinity for CsA. The presence of the mitochondrially-restricted PPIase that was believed to be responsible for the CsA-sensitive mPTP-blocking activity was confirmed in 1992 when this cyclophilin, now known as CypD, was first purified from isolated liver mitochondria (Connern & Halestrap, 1992).

The relationship between CypD and mPTP formation was then confirmed following the generation of the CypD-knockout mice (Baines et al., 2005). *In vitro*, formation of the mPTP requires high levels of intracellular Ca$^{2+}$ (Gunter & Pfeiffer, 1990); however proximal tubule cells from mice lacking CypD protein display a striking desensitization of the mPTP in response to Ca$^{2+}$, and they behave identically to control mitochondria that were treated with CsA (Devalaraja-Narashimha et al., 2009). Oxidative stress is also known to induce mPTP formation; however, *in vitro* it has been shown that
CypD deficient murine embryonic fibroblast cells are resistant to H₂O₂-induced cell death in comparison to wild type cells (Nakagawa et al., 2005). Thus, active CypD acts as a primary positive regulator of mitochondrial permeability transition in response to cell stressors such as Ca²⁺ and oxidative stress.


Mice lacking CypD expression are viable and fertile (Basso et al., 2005), thus the role of the mPTP is not prominent within the mitochondria under steady state. These CypD-knockout mice, however, have been shown to be resistant to a number of acute ischemic injuries, including in the liver, muscle, heart, brain, and kidney (Devalaraja-Narashimha et al., 2009; Doran & Halestrap, 2000; Duchen, McGuinness, Brown, &
Crompton, 1993; Nakayama et al., 2007; Park, Pasupulati, Feldkamp, Roeser, & Weinberg, 2011; Schinzel et al., 2005). In acute kidney injury (AKI), which results from impaired blood flow, an ischemic cascade leads to disruption of calcium homeostasis, increased oxidative stress, depletion of intracellular ATP, endothelial injury, and pericyte detachment and differentiation into myofibroblasts (Kalogeris, Baines, Krenz, & Korthuis, 2012; Kramann & Humphreys, 2014). In a mouse model of AKI, ischemia reperfusion injury (IRI), proximal tubules show a bioenergetic deficit independent of CypD activity. However, this bioenergetic deficit predisposes these epithelial cells to later develop mPTP formation leading to necrosis (Park et al., 2011), which contributes pathologically to AKI. Importantly, the latter is evidenced by protection in CypD-knockout mice subjected to IRI (Devalaraja-Narashimha et al., 2009). Specifically, knockout mice demonstrated functional protection in that they had significantly decreased blood urea nitrogen and serum creatinine levels as compared to post-IRI wild type mice. Furthermore, tissue injury and necrotic cell death were also reduced in the knockout mice, as quantified by a reduction in tubular cast formation and cellular necrosis. These data thus suggest the therapeutic potential of mPTP blockade via pharmacological inhibition of CypD as an approach to obtain functional and morphological protection in AKI.

Recent studies have also shown that blocking mPTP formation, either genetically by CypD deletion or pharmacologically using CsA or other cyclophilin-specific inhibitors, can be a compelling option in settings of chronic injury as well. In a mouse model of heart failure, induced by enhanced sarcolemmal L-type Ca^{2+} channel activity and subsequent intracellular Ca^{2+} overload, genetic deletion of CypD expression blocked
onset of heart failure. Histologically, heart tissue from these mice showed less fibrosis and myocyte dropout than wild type controls. Additionally, mice devoid of CypD expression had a 100% survival rate in this model of heart failure (Nakayama et al., 2007). Similar benefit was also seen in two distinct models of muscular dystrophy, whereby genetic deletion of CypD culminated in less dystrophic disease in both skeletal muscle and heart, as well as a rescue from premature lethality (Merlini et al., 2008; Millay et al., 2008). These results were further supported when mice from a model of muscular dystrophy were treated with Debio-025, a cyclophilin inhibitor, and showed noticeable improvement in myofiber organization and a significant reduction in fibrosis in the diaphragm, gastrocnemius, and quadriceps (Millay et al., 2008). While studies with CypD-knockout mice have not been reported in models of CKD, these results in models of chronic cardiac and skeletal muscle injury support that treatment with mPTP inhibitors represents a promising option for numerous mitochondrial-driven chronic diseases.

Cyclosporine A in Chronic Kidney Disease

CsA is a powerful immunosuppressant used frequently in organ transplants to prevent rejection; however, it has also been shown to inhibit mPTP formation through binding to and preventing the activity of CypD (Baines et al., 2005; Basso et al., 2005; Broekemeier, Dempsey, & Pfeiffer, 1989; Crompton, Ellinger, & Costi, 1988; Fournier, Ducet, & Crevat, 1987; Gunter & Pfeiffer, 1990; Halestrap & Davidson, 1990). Numerous studies have shown that human Alport nephropathy patients treated with CsA demonstrate resolution of proteinuria for up to 10 years during continuous treatment (Callis, Vila, Carrera, & Nieto, 1999). Furthermore, a canine model of Alport syndrome revealed that treatment with CsA delayed progression of multilaminar changes to the
glomerular basement membrane, as well as showed improvements to weight gain, serum creatinine levels, and glomerular filtration rate. However, the authors noted no significant differences in interstitial fibrosis or proteinuria, thus suggesting that CsA may slow rather than halt progression of disease in this model (Chen et al., 2003).

Since CsA is a strong immunosuppressant as well as a nephrotoxin (Calne et al., 1978), further studies must be performed to dissect its mPTP-blocking activities from its immunosuppressive role, while also seeking alternative inhibitors with improved safety profiles.

Inhibition of the mPTP by Targeting CypD as a Novel Therapy for CKD

Prior studies have suggested that pharmacologically blocking mPTP formation may be a compelling option for treating CKD; however the efficacy of specifically inhibiting mPTP formation by targeting CypD in CKD has yet to be established. In this study, we use a mouse model of Alport nephropathy (Col4a3^−/−) that features mitochondrial dysfunction to determine the efficacy of inhibiting mPTP formation by genetic deletion of CypD. We hypothesized that this would limit the mitochondrial stress within the proximal tubule and thereby limit signals from stressed proximal tubules to pericytes, thus abrogating chronic renal fibrotic disease onset and/or progression (Figure 8). Although CKD in human patients arises from different etiologies, including hypertension, diabetes mellitus, and vascular disease, they are believed to progress to end stage renal disease through similar mechanisms. The use of a mouse model of Alport nephropathy in this study is advantageous in that it lacks the comorbid conditions often associated with other CKD populations and always progresses to end stage renal disease.
Here, we report that genetic deletion of CypD (Ppif<sup>−/−</sup>) expression, as an approach to inhibit mPTP formation, in a mouse model of CKD (Alport nephropathy) is insufficient to prevent or delay disease onset or reduce disease severity.

Figure 8. Hypothetical Outcome of Inhibiting CypD-Dependent mPTP Formation in CKD. In our hypothesized model, inhibition of mPTP formation via the full or partial deletion of CypD expression results in decreased mitochondrial stress, limiting of proximal tubule cross talk with nearby pericytes, and thus abrogation of CKD onset/progression.
Chapter II.
Materials and Methods

The following section details the materials and techniques used throughout the study. Briefly, human kidney tissue and murine kidney tissue were used to measure alterations in mitochondria in CKD as compared to healthy control tissue. Next, using Col4a3⁻/⁻ and another previously described genetically modified mouse strain, Ppif⁻/⁻, a novel cross was made that allowed us to ablate CypD expression in a mouse model of Alport nephropathy. In so doing, we prevented CypD-sensitive mPTP formation in a chronic kidney disease setting. By monitoring animal wellbeing, measuring renal function, and conducting a histological analysis of kidney tissue taken from these mice, we characterized the value of inhibiting CypD, as an approach to block mPTP formation, as a potential therapeutic target.

Human Kidney Tissue Assessment of Mitochondrial Morphology and Protein Expression in Health and CKD

In order to obtain human renal tissue for histological analysis of changes in mitochondrial morphology, kidney samples were collected from adult patients undergoing partial nephrectomy to remove renal carcinoma at Tufts New England Medical Center (Boston, MA) under a research agreement. All patients consented to the removal of a small portion of non-cancerous kidney tissue during their partial nephrectomy and also consented to the use of their discarded tissue for research purposes. All samples were de-identified. A pathologist scored the kidney tissue for the presence of
disease, and basic clinical and laboratory data were provided. Adult human kidney tissue samples from both patients exhibiting clinical signs of diabetic nephropathy (DN) or FSGS and control patients were obtained. Upon delivery, samples were fixed for two hours in a periodate-lysine-paraformaldehyde solution, immersed in 18% sucrose in PBS for sixteen hours, and then embedded and frozen in OCT. To detect antigens in 6 μm sections, a primary antibody against the following protein was used for labeling: SDHA [clone EPR9043(B); Abcam], a subunit of complex II of the ETC used as a marker of mitochondrial morphology (Table 1). Fluorescent-conjugated affinity-purified secondary antibody labeling (1:400; Jackson ImmunoResearch) was performed. Sections were colabeled with DAPI and mounted with ProLong Gold (Thermo Fisher). For each individual patient sample, ten 63x confocal images of kidney cortex were captured, one section per sample, by confocal microscopy and cells identified by blue DAPI staining. Images were analyzed using a custom MATLAB (Natick, MA) script. The nuclear signal from the blue channel (DAPI) was compared to a threshold and then filtered by a size constraint. SDHA-positive cells in the red channel (Alexa Fluor 594) were then analyzed using a mask created by each nucleus to determine the fluorescent signal associated with each cell. By comparing the mean cell signal to a predetermined threshold, the number of positive pixels associated with a nucleus was determined, reported as SDHA+ area (pixels). The ten values were averaged for each animal to give the reported value.

To obtain protein for western blot analysis of mitochondrial ETC and ROS scavenging proteins from human proximal tubule epithelial cells (PTECs), human PTEC purification was performed by preparing single cell digest from healthy control, CKD (Stage 1), and CKD (Stage 5) kidneys, as described (Ren et al., 2013), with
modifications. Briefly, kidneys were minced and then incubated in a shaking water bath (180 rpm) at 37°C for 50 min with Liberase DL (0.5 mg/mL; Roche) and DNase (100 U/mL; Roche) in serum-free DMEM/F12. The tissue digest was mixed thoroughly and filtered (40 μm). Proximal tubule epithelial cells were separated from the single cell suspension using the Dynabead magnetic bead conjugation system, whereby lotus tetragonolobus lectin (LTL), a known marker for proximal tubule, which is supplied in biotinylated form (Vector Laboratories), was incubated with Dynabeads Biotin Binder (Thermo Fisher). For each kidney isolate, 100 μL beads were conjugated with 40 μg LTL at 4°C for 45 min. Beads were then washed twice with MACS buffer (Miltenyi Biotec) and incubated with the single cells suspension in a final volume of 1 mL at 4°C for 45 min. Beads and proximal tubule cells were then trapped against a magnet and residual non-proximal-tubule-cells were washed away. Isolated renal PTECs were harvested immediately for protein analysis in ice-cold RIPA cell lysis buffer (Boston Bioproducts) with HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). Samples were centrifuged for 5 minutes at 14,000 x g, and the supernatant was taken for protein determination. Cell extracts containing 15 μg protein were prepared in SDS sample buffer and subjected to SDS-PAGE. After transfer to nitrocellulose paper and blocking, immunostaining was performed in TBST buffer. The following antibodies were used to detect the specific proteins: Oxidative Stress Defense cocktail (Catalase, SOD1, & Thioredoxin; Abcam), mitochondrial-associated ROS scavenging proteins; SOD2 (polyclonal, Abcam), a mitochondrial-restricted antioxidant enzyme; SDHA [clone EPR9043(b); Abcam], the major catalytic subunit of Complex II of the ETC; COX10 (polyclonal, MyBioSource), a nuclear DNA-encoded protein required for the expression
of functional Complex IV of the ETC; COX1 (polyclonal, MyBioSource), a mtDNA-encoded protein that functions as the catalytic subunit of Complex IV of the ETC; β-actin (clone C-11; Santa Cruz Biotechnology), which acts as the loading control (Table 1).

Bound primary antibodies were labeled with HRP-conjugated anti-rabbit or anti-mouse IgG antisera (GE Healthcare). Positive bands were detected by enhanced chemiluminescence (ECL, Pierce; SuperSignal West Dura; Thermo Scientific), and luminescence was captured by the ChemiDoc MP Imaging System (Bio-Rad). Densitometry quantification was performed in ImageJ software.

Table 1: Human Immunofluorescent Stains and Western Blots

<table>
<thead>
<tr>
<th>Cell Marker</th>
<th>Assay*</th>
<th>Goal of Antibody Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>WB</td>
<td>Loading control used to assess normalized western blots.</td>
</tr>
<tr>
<td>Catalase</td>
<td>WB</td>
<td>A key antioxidant enzyme in the defense against oxidative stress that converts hydrogen peroxide to water and oxygen (see ‘SOD2’).</td>
</tr>
<tr>
<td>COX1</td>
<td>WB</td>
<td>A mtDNA-encoded protein that functions as the catalytic subunit of Complex IV of the ETC (see ‘COX10’).</td>
</tr>
<tr>
<td>COX10</td>
<td>WB</td>
<td>A nuclear DNA-encoded protein required for the expression of functional Complex IV of the ETC (see ‘COX1’).</td>
</tr>
<tr>
<td>SDHA</td>
<td>IHF</td>
<td>Subunit of Complex II of the ETC used as a marker of mitochondrial morphology.</td>
</tr>
<tr>
<td>SOD1</td>
<td>WB</td>
<td>An enzyme responsible for destroying free superoxide radicals.</td>
</tr>
<tr>
<td>SOD2</td>
<td>WB</td>
<td>A mitochondrially-restricted protein that converts superoxide byproducts of the ETC to hydrogen peroxide and diatomic oxygen (see ‘Catalase’).</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>WB</td>
<td>An enzyme involved in numerous redox reactions, contributing to the response to intracellular nitric oxide.</td>
</tr>
</tbody>
</table>

* WB: Western Blot for protein expression levels | IHF: Immunohistofluorescence stain for morphology

Mouse Strains

Col4a3+/- mice on a 129X1/SvJ genetic background, acquired from the original generating authors (Kang et al., 2006), were obtained from heterozygous breeding after
mutant mice on the SJL background were crossed to 129X1/SvJ mice for 9 generations. *Col4a3*<sup>−/−</sup> mice and littermate controls were described previously and genotyped by methods described (Gomez et al., 2015).

*Ppif*<sup>+/−</sup> mice were obtained from The Jackson Laboratory, where they are maintained on a mixed C57BL/6 and 129 genetic background. *Ppif*<sup>−/−</sup> mice and littermate controls were described previously and genotyped by methods described (Baines et al., 2005).

All animal studies were performed under protocols approved by the Biogen IACUC review board (protocol 0489-2013).

Mouse Kidney Tissue Assessment of Tissue Pathology and Mitochondrial Morphology

In order to determine changes in mitochondrial morphology prior to and following disease onset, kidney tissue from *Col4a3*<sup>+/+</sup> (8 weeks of age) and *Col4a3*<sup>−/−</sup> (4 weeks and 8 weeks of age) littermate mice on a pure 129X1/SvJ genetic background were euthanized at the ages indicated and the mice were perfused with ice cold PBS to flush the blood from the tissue. We then extracted the kidneys, removed the capsules from them, and segmented the kidneys sagitally.

One half kidney was fixed for two hours in a periodate-lysine-paraformaldehyde solution, immersed in 18% sucrose in PBS for sixteen hours, and then embedded and frozen in OCT. To detect antigens in 6 μm sections, primary antibodies against the following proteins were used for labeling: SDHA [clone EPR9043(B); Abcam], a subunit of complex II of the ETC used as a marker of mitochondrial morphology (Table 1). Fluorescent-conjugated affinity-purified secondary antibody labeling (1:400; Jackson
ImmunoResearch) was performed. Sections were colabeled with DAPI and mounted with ProLong Gold (Thermo Fisher). For each individual animal, ten 63x confocal images of kidney cortex were captured, one section per animal per stain, by confocal microscopy and cells identified by blue DAPI staining. Images were analyzed using a custom MATLAB (Natick, MA) script. The nuclear signal from the blue channel (DAPI) was compared to a threshold and then filtered by a size constraint. SDHA-positive cells in the red channel (Alexa Fluor 594) were then analyzed using a mask created by each nucleus to determine the fluorescent signal associated with each cell. By comparing the mean cell signal to a predetermined threshold, the number of positive pixels associated with a nucleus was determined, reported as SDHA$^+$ area (pixels). Additionally, by comparing the average pixel intensity in the region of interest (positive cells), the mean fluorescence intensity (MFI) was also calculated. For each measurement, the ten respective values were averaged for each animal to give the reported values.

One half kidney was fixed in 10% neutral buffered formalin for sixteen hours and transferred to 70% ethanol before being processed for embedding into paraffin wax. Epithelial injury was assessed in PAS stained kidney sections as described (Ren et al., 2013).

Mouse Renal Proximal Tubule Cell Isolation and Assessment of mtDNA Copy Number and Mitochondrial ETC Protein Expression

Mouse renal proximal tubule cell purification was performed by preparing single cell digest from Col4a3$^{+/+}$ and Col4a3$^{-/-}$ littermate mouse kidneys at 4 and 8 weeks of age, as described (Ren et al., 2013), with modifications. Briefly, kidneys were decapsulated, minced, and then incubated in a shaking water bath (180 rpm) at 37°C for
50 min with Liberase DL (0.5 mg/mL; Roche) and DNase (100 U/mL; Roche) in serum-
free DMEM/F12. The tissue digest was mixed thoroughly and filtered (40 μm). Proximal
tubule epithelial cells were separated from the single cell suspension using the Dynabead
magnetic bead conjugation system, whereby lotus tetragonolobus lectin (LTL), a known
marker for proximal tubule, that is supplied in biotinylated form (Vector Laboratories),
was incubated with Dynabeads Biotin Binder (Thermo Fisher). For each kidney isolate,
100 μL beads were conjugated with 40 μg LTL at 4°C for 45 min. Beads were then
washed twice with MACS buffer (Miltenyi Biotec) and incubated with the single cells
suspension in a final volume of 1 mL at 4°C for 45 min. Beads and proximal tubule cells
were then trapped against a magnet and residual non-proximal-tubule-cells were washed
away.

Isolated PTECs were harvested immediately for DNA analysis using the
QIAquick Purification Kit (QIAGEN). Subsequently, the purified DNA was analyzed
using the NovaQUANT Mouse Mitochondrial to Nuclear Ratio Kit (EMD Millipore),
which compares the levels of nuclear DNA to mtDNA in a mouse DNA sample. Briefly,
the purified DNA was quantified and a sample mix was prepared by combining
DNase/RNase-free DNA (2 ng per well), water, and qPCR iTaq Universal SYBR Green
Supermix (Bio-Rad). This sample mix was then aliquoted into the kit-provided qPCR
plate, which contains four optimized PCR primer pairs targeting two mitochondrial genes
(trLEV and 12s RNA) and two nuclear genes (BECN1 and NEB). A fast real-time qPCR
system (Applied Biosystems) was used to measure the ratio of mtDNA to nuclear DNA
(relative mtDNA copy number), which reflects the average relative mtDNA content per
cell. The results of the qPCR reactions were analyzed with $2^{-\Delta CT}$ method according to manufacturer instruction.

To obtain protein for western blot analysis of mitochondrial ETC proteins from mouse PTECs, purification of PTECs was performed as described above. Freshly isolated PTECs were lysed in ice-cold RIPA cell lysis buffer (Boston Bioproducts) with HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). Samples were centrifuged for 5 minutes at 14,000 x g, and the supernatant was taken for protein determination. Cell extracts containing 15 μg protein were prepared in SDS sample buffer and subjected to SDS-PAGE. After transfer to nitrocellulose paper and blocking, immunostaining was performed in TBST buffer. The following antibodies were used to detect the specific proteins: Total OXPHOS Rodent WB Antibody Cocktail (Abcam) for analysis of the relative levels of the five ETC complexes (Table 2). Bound primary antibodies were labeled with HRP-conjugated anti-mouse IgG antisera (GE Healthcare). Positive bands were detected by enhanced chemiluminescence (ECL, Pierce; SuperSignal West Dura; Thermo Scientific), and luminescence was captured by the ChemiDoc MP Imaging System (Bio-Rad).

Table 2: Detailed Description of Electron Transport Chain Components and Their Encoding DNA Locations Used in Western Blot Analysis to Determine Alterations in the Relative Levels of the Five ETC Complexes

<table>
<thead>
<tr>
<th>ETC Complex Represented</th>
<th>Complex Subunit Recognized</th>
<th>DNA Expression: Nuclear DNA vs. mtDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>NDUFB8</td>
<td>Nuclear DNA</td>
</tr>
<tr>
<td>Complex II</td>
<td>SDHB</td>
<td>Nuclear DNA</td>
</tr>
<tr>
<td>Complex III</td>
<td>UQRC2</td>
<td>Nuclear DNA</td>
</tr>
<tr>
<td>Complex IV</td>
<td>MTCO1</td>
<td>mtDNA</td>
</tr>
<tr>
<td>Complex V</td>
<td>ATP5A</td>
<td>Nuclear DNA</td>
</tr>
</tbody>
</table>
Breeding Scheme to Generate Experimental Animals:

Alport Nephropathy Mice with Ppif Sufficiency or Deficiency

A breeding scheme was undertaken to generate the five necessary genotypes for these experiments (see Table 3): (1) Ppif<sup>+/+</sup>;Col4a3<sup>-/-</sup>, (2) Ppif<sup>+/+</sup>;Col4a3<sup>-/-</sup>, (3) Ppif<sup>-/-</sup>;Col4a3<sup>-/-</sup>, (4) Ppif<sup>+/+</sup>;Col4a3<sup>+/+</sup>, and (5) Ppif<sup>-/-</sup>;Col4a3<sup>+/+</sup>.

We first crossed Ppif<sup>+/+</sup>;Col4a3<sup>+/-</sup> (129X1;SvJ-Col4a3) mice with Ppif<sup>-/-</sup>;Col4a3<sup>+/+</sup> (B6;129-Ppiftm1Jmol/J) mice. From this cross, we used the resulting litters for the second round of breeding, selecting Ppif<sup>+/+</sup>;Col4a3<sup>-/-</sup> males to breed with Ppif<sup>-/-</sup>;Col4a3<sup>-/-</sup> females in a trio breeding format. Mice of the five genotypes listed above were successfully bred (Charles River Laboratories; Wilmington, MA), as verified by western blot (Figure 11).

Assessment of Cyclophilin D Protein Expression in Experimental Animals

To verify the partial or full deficiency of Ppif protein expression (CypD), snap-frozen kidney tissue was obtained for western blot analysis. Snap-frozen kidneys harvested from 12-week-old mice were lysed in ice-cold RIPA cell lysis buffer (Boston Bioproducts) with HALT protease and phosphatase inhibitor cocktail (Thermo Scientific), using the Omni Bead Ruptor to homogenize the tissue. Samples were centrifuged for 5 minutes at 14,000 g, and supernatant was taken for protein determination. Cell extracts containing 15 μg protein were prepared in SDS sample buffer and subjected to SDS-PAGE. After transfer to nitrocellulose paper and blocking,
immunostaining was performed in TBST buffer. The following antibody was used to
detect specific proteins: Cyclophilin D (1:1000, Abcam, ab110324, clone E11AE12BD4),
β-Actin (C4) which acts as the loading control (1:1000, Santa Cruz, sc-47778). Bound
primary antibodies were labeled with HRP-conjugated anti-mouse IgG antisera (1:3000,
GE Healthcare). Positive bands were detected by enhanced chemiluminescence (ECL,
Pierce; Thermo Fisher), and luminescence was captured by the G:BOX (Syngene).
Densitometry quantification was performed in ImageJ software.

Experimental Design for Analysis of Alport Nephropathy Mice with \textit{Ppif} Sufficiency or
Deficiency

Previous reports indicated that the \textit{Col4a3}\textsuperscript{-/-} (129X1;SvJ -\textit{Col4a3}) male mice
reach peak body mass and begin to lose weight between 6-8 weeks of age, whereas their
wild type counterparts do not lose weight within the first year (Gomez et al., 2015).
Given that we crossed these mice with the \textit{Ppif}\textsuperscript{-/-} (B6;129-\textit{Ppif}\textsuperscript{tm1Jmol/J}) strain, which
are on a mixed strain genetic background, it was anticipated that the timing of disease
onset and weight loss might be different. Furthermore, due to the mixed background of
experimental mice, the progression and severity of disease might also be less consistent
between individual mice of a given experimental group. We therefore enrolled a greater
number of mice in each group (n=25 total mice per genotype: 10 mice for survival studies
and 15 mice for histological assessment) as compared to prior studies using the Alport
strain (n=3-12 mice/group). To fulfill the needs for this study, we began production of
mice in August of 2016 and continued to acquire mice for each genotype on a rolling
basis until June of 2017, at which point the study’s animal requirements had been achieved.

As conveyed in Table 3, we used \( Ppi{f}^{+/+};Col4a3^{-/-} \) male mice as controls to determine normal progression of the disease in mice wild type for CypD. We used \( Ppi{f}^{-/-};Col4a3^{+/+} \) and \( Ppi{f}^{+/+};Col4a3^{+/+} \) male mice as controls to track healthy mice that are wild type for \( Col4a3 \). We then used \( Ppi{f}^{+/+};Col4a3^{+/+} \) male mice as our experimental group to determine whether a complete ablation of CypD is sufficient to prevent progression of Alport nephropathy. Finally, we used \( Ppi{f}^{+/+};Col4a3^{-/-} \) male mice to determine whether a partial (heterozygous) deletion of CypD could also provide a measurable level of protection from disease progression.

Table 3. Description of Strain Genotypes

<table>
<thead>
<tr>
<th>( Ppi{f} )</th>
<th>( Col4a3 )</th>
<th>Experimental Group Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>Wild type control</td>
</tr>
<tr>
<td>-/-</td>
<td>+/+</td>
<td>Demonstrates effect of CypD deletion in mice wild type for ( Col4a3 )</td>
</tr>
<tr>
<td>+/+</td>
<td>-/-</td>
<td>Determines normal progression of disease in mice wild type for CypD and lacking ( Col4a3 )</td>
</tr>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>Demonstrates whether partial loss of CypD has any effect on disease in mice lacking ( Col4a3 )</td>
</tr>
<tr>
<td>-/-</td>
<td>-/-</td>
<td>Demonstrates whether total loss of CypD ameliorates disease in mice lacking ( Col4a3 )</td>
</tr>
</tbody>
</table>

Wellbeing, Weight, and Survival Assessment of Alport Nephropathy Mice with \( Ppi{f} \) Sufficiency or Deficiency

In order to evaluate survival in the various genotypes in our study, we allowed \( n=10 \) mice per genotype to age while we monitored them at least twice weekly (starting
at 7 wks through 16 wks of age) for body weight, body condition scoring (BCS) (Table 4), and Animal Health Assessment. The latter is a descriptive assessment, which is indicative of declining health, and includes inspecting for signs of lethargy, moribund behavior, dehydration, abnormal locomotion, and ruffled fur. If animals lost weight (15% from peak body weight), then we monitored them daily. However, if an animal reached over 20% body weight loss or showed a poor body condition score of less than or equal to two, or if the animal was moribund (as defined by a general unresponsiveness and lack of awareness of external stimuli), the mouse was euthanized immediately and the date of death recorded in order to form the survival curve.

Table 4. Body Conditioning Score Chart

<table>
<thead>
<tr>
<th>BCS</th>
<th>Scoring Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mouse is emaciated:</td>
</tr>
<tr>
<td></td>
<td>Skeletal structure extremely prominent; little or no flesh cover. Vertebrae</td>
</tr>
<tr>
<td></td>
<td>distinctly segmented.</td>
</tr>
<tr>
<td>2</td>
<td>Mouse is underconditioned:</td>
</tr>
<tr>
<td></td>
<td>Segmentation of vertebral column evident. Dorsal pelvic bones are readily palpable.</td>
</tr>
<tr>
<td>3</td>
<td>Mouse is well-conditioned:</td>
</tr>
<tr>
<td></td>
<td>Vertebrae and dorsal pelvis not prominent; palpable with slight pressure.</td>
</tr>
<tr>
<td>4</td>
<td>Mouse is overconditioned:</td>
</tr>
<tr>
<td></td>
<td>Spine is a continuous column. Vertebrae palpable only with firm pressure.</td>
</tr>
<tr>
<td>5</td>
<td>Mouse is obese:</td>
</tr>
<tr>
<td></td>
<td>Mouse is smooth and bulky. Bone structure disappears under flesh and subcutaneous</td>
</tr>
<tr>
<td></td>
<td>fat.</td>
</tr>
</tbody>
</table>

Kidney Function Evaluation of Alport Nephropathy Mice with Ppif Sufficiency or Deficiency

To measure kidney function, timed urine collections were performed the day prior to necropsy and blood serum was collected at the time of necropsy (12 weeks of age). Plasma and urine creatinine levels (mg/dL) were measured using Creatinine Liquid
Reagents Assay (DIAZYME). Urine albumin concentration was measured using Albuwell M Kit (Exocell) and was normalized to urine creatinine levels (mg/gCr). Urea and nitrogen levels in the blood serum (mg/dL) were detected by BUN Reagent Set (Pointe Scientific).

Histological Assessment of Alport Nephropathy Mice with Ppif Sufficiency or Deficiency

In order to analyze the histopathological changes associated with each genotype, fifteen mice per group were allowed to age to 12 weeks. At that point, we performed a cardiac perfusion with ice cold PBS to flush the blood from the tissue. We then extracted the kidneys and removed the capsules from them and segmented the kidneys sagitally.

For immunofluorescent stains measuring myofibroblast expansion, capillary density, and leukocyte infiltration, one half kidney was fixed for two hours in a periodate-lysine-paraformaldehyde solution, immersed in 18% sucrose in PBS for sixteen hours, and then embedded and frozen in OCT. To detect antigens in 6 μm sections, primary antibodies against the following proteins were used for labeling: αSMA-Cy3 which detects myofibroblasts (clone 1A4; Sigma), CD31 which detects endothelial cells (clone MEC 13.3; BD Pharmingen), CD11b which detects leukocytes (clone M1/70; Thermo Fisher), and Cleaved Caspase-3 (Asp175) which detects cells undergoing apoptosis (clone 5A1E; Cell Signaling). Fluorescent-conjugated affinity-purified secondary antibody labeling (1:400; Jackson ImmunoResearch) was performed where indicated. Sections were colabeled with DAPI and mounted with ProLong Gold (Thermo Fisher). For each individual animal, five 40x confocal images of kidney cortex were captured, one section per animal per stain, by confocal microscopy and cells identified by blue DAPI
staining. Based on fluorescence intensities ranging from 0-255 in ImageJ, myofibroblasts (αSMA), peritubular capillaries (CD31), and leukocytes (CD11b) were distinguished from background by empirically determined threshold values that marked only labeled cells (Table 5). The threshold was constant for all measurements. Captured images were quantified morphometrically to measure area of fluorescent signal using ImageJ and the five values were averaged for each animal to give the reported value. Each immunofluorescent stain was performed on a non-contiguous section.

Table 5. Purpose of Mouse Immunofluorescent Stains

<table>
<thead>
<tr>
<th>Cell Marker</th>
<th>Goal of Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA</td>
<td>Marker of myofibroblast expansion:</td>
</tr>
<tr>
<td></td>
<td>Measured by % area</td>
</tr>
<tr>
<td>CD31</td>
<td>Marker of capillary density:</td>
</tr>
<tr>
<td></td>
<td>Measured by % area</td>
</tr>
<tr>
<td>CD11b</td>
<td>Marker of leukocyte infiltration:</td>
</tr>
<tr>
<td></td>
<td>Measured by % area</td>
</tr>
<tr>
<td>Cleaved Caspase-3</td>
<td>Marker of cells undergoing apoptosis:</td>
</tr>
<tr>
<td></td>
<td>Images demonstrate presence/absence of apoptotic tubules</td>
</tr>
</tbody>
</table>

For histological staining for interstitial fibrosis, the other half kidney was fixed in 10% neutral buffered formalin for sixteen hours and transferred to 70% ethanol before being processed for embedding into paraffin wax. Interstitial fibrosis analysis was performed and quantified by the University of Washington Department of Pathology (Seattle, WA). Paraffin-embedded samples were sectioned at 4 μm and stained in picosirius red. To quantify the percent area of collagen by picosirius red staining, five 40x images were taken for each animal using a polarized light microscope and percent area was quantified by Photoshop (Adobe). The five values were averaged for each animal to give the reported value.
Kidney histopathology analysis was performed and quantified by Arkana Laboratories (Little Rock, Arkansas). Four micrometer sections stained with periodic-acid Shiff (PAS) were examined at 200x and 400x using an Olympus BX51 microscope (Olympus Corp. Tokyo, Japan). A total of 75 glomeruli were analyzed on one section for each sample. Specific lesions involving the glomeruli were noted, including global glomerulosclerosis, segmental glomerulosclerosis, mesangial hypercellularity/proliferation, fibrinoid necrosis, and crescentic formation. The glomerular changes described above were defined according to routine renal biopsy practice as follows: Focal, involving <50% of the glomerulus; Diffuse, involving >50% of the glomerulus; Segmental, lesion involving <50% of the glomerular area in involved glomerulus; Global, lesion involving >50% of the glomerular area in involved glomerulus; Global glomerulosclerosis, sclerosis of the entire glomerulus (obliteration of the capillary lumen by increased extracellular matrix with or without hyalinosis or foam cells); FSGS, <50% of the glomerulus demonstrate sclerosis involving <50% of the glomerular area; Mesangial hypercellularity, >3 mesangial cells per mesangial region in a 4 micron thick section; Crescents, extracapillary proliferation of >2 cell layers occupying 25% or more of the extracapillary space beneath Bowman’s capsule; Fibrinoid necrosis, lesion characterized by fragmentation of nuclei or disruption of capillary loops with the presence of fibrin-rich material.

Epithelial injury was quantified in PAS-stained kidney sections as described (Ren et al., 2013). Briefly, five 200x images per specimen were scored to determine the percent of tubules showing signs of tubular injury (i.e. tubular dilation, cellular necrosis, nuclear dropout, and/or the loss of defined brush border membranes). The average of
these five values was calculated for each specimen and the mean values for each cohort reported as a percentage on a score of 0-100%.

Statistics

Data are presented as mean ± SEM. Differences between multiple groups were assessed by 1-way ANOVA with Dunnett’s post-hoc test or by 2-way ANOVA with Tukey’s post-hoc test. Differences in survival were assessed by Gehan-Breslow-Wilcoxon test. $P < 0.05$ was considered significant.
Chapter III.

Results

Kidney tissue from mice and humans with chronic kidney disease were collected and investigated for signs of mitochondrial dysfunction that could be indicative of mPTP formation. Mice lacking CypD and COL4a3 protein were successfully bred and data collected from this novel cross was used to determine whether inhibition of CypD activity was sufficient to ameliorate disease progression in a model of Alport nephropathy. The data collected from these experiments are described in further detail in this section.

Human CKD is Associated with Mitochondrial Alterations

To study the effect of disease on mitochondria in human CKD patients, tissue samples from controls, FSGS, and DN patients were obtained. Immunofluorescent staining for SDHA, a component of the ETC restricted to the inner mitochondrial membrane, primarily stains the tubular epithelial cells due to the abundance of mitochondria within these cells. The mitochondrial morphology in FSGS and DN tissues, as revealed by pattern of expression of SDHA in the renal tubules, was significantly different than controls (Figure 9, A-B). While in control samples the mitochondria are abundant and involved in extensive mitochondrial networking, both the FSGS and DN
samples showed a loss of the mitochondrial network and/or fewer mitochondria, as quantified by pixel area.

To study the effect of disease on expression of ROS scavenging proteins and ETC components in renal PTECs, western blot analysis was performed using protein from PTECs isolated from healthy human control and CKD patients (Stage 1 & Stage 5). As shown in Figure 9 C-D, there was no significant difference on any measured protein between the healthy control and CKD Stage 1 patients. However, numerous differences were found when comparing healthy control to the CKD Stage 5 patient samples. In the human CKD Stage 5 PTECs, there was a significant increase in the following proteins: SDHA ($P = 0.0227$), a nuclear DNA-encoded subunit of complex II of the ETC; COX10 ($P < 0.0001$), a nuclear DNA-encoded protein required for the expression of functional complex IV of the ETC. The SDHA results were particularly striking, given the observed aberrant mitochondria of CKD patients by SDHA immunofluorescent staining (Figure 9A). In the human CKD Stage 5 PTECs, there was also a significant decrease in the expression of SOD2 ($P = 0.0002$), a mitochondrially-restricted protein that converts superoxide byproducts of the ETC to hydrogen peroxide and diatomic oxygen. Though not statistically significant, there was also a trend towards a decrease in the expression in the following proteins: COX1 ($P = 0.0664$), a mtDNA-encoded protein that functions as the catalytic subunit of Complex IV of the ETC; Thioredoxin ($P = 0.0753$), an enzyme involved in numerous redox reactions that contributes to the response to intracellular nitric oxide. Finally, there was no significant difference in the expression of SOD1 or Catalase, two key antioxidant enzymes in the defense against oxidative stress, which is
Figure 9. Kidney samples from CKD patients show differences in mitochondrial morphology and ETC protein levels as compared to healthy controls. (A) Images of mitochondrial morphology (SDHA). (B) Quantification of pixel area positive for SDHA. (C) Western blots showing effect of disease on expression of human ETC components (SDHA, COX10, COX1) and ROS scavenging proteins (SOD1, SOD2, Catalase, Thioredoxin), compared to control (β-Actin), in isolated proximal tubule epithelial cells. (D) Densitometric analysis of SDHA, COX10, COX1, SOD1, SOD2, Catalase, and Thioredoxin normalized to β-Actin. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, by 2-Way ANOVA with Tukey’s post-hoc test.
striking given the increased levels of ROS present in human CKD kidneys (Zhang et al., 2012).

Alport Nephropathy Results in Early Mitochondrial Changes in Mice

To study the effect of disease in a mouse model of Alport nephropathy, renal tissue was collected from 129X1/SvJ background Col4a3+/+ mice at 8 weeks of age and from littermate Col4a3−/− mice at 4 and 8 weeks of age. By analyzing for histological signs of disease by PAS, Col4a3−/− mice at 4 weeks of age show essentially normal histology, though by 8 weeks of age these mice demonstrate clear signs of tubule injury and glomerulosclerosis (Figure 10A), which is in accordance with previous findings (Gomez et al., 2015). When comparing the expression of the mitochondrial marker SDHA however, it becomes apparent there are changes as early as 4 weeks of age in diseased samples. As shown by immunofluorescent staining of SDHA, a component of the ETC restricted to the inner mitochondrial membrane, both Col4a3−/− mice at age 4 weeks and 8 weeks have mitochondrial puncta within their tubules (white arrowheads), as indicated by increased MFI (Figure 10C), which signify mitochondrial fusion, a sign of mitochondrial stress. Additionally, there is also a significant loss of mitochondrial networking by 8 weeks of age, as indicated by the decrease in SDHA staining area (Figure 10B).

Mitochondrial dysfunction is characterized by mitochondrial fragmentation and the loss of mtDNA integrity (Zhan, Brooks, Liu, Sun, & Dong, 2013). The mtDNA copy number is considered a critical component of overall mitochondrial health as reduced mtDNA content may contribute to decreased mitochondrial functionality and has been associated with decreased renal function (Lee et al., 2009). To study the effect of disease
Figure 10. Murine Col4a3−/− kidney samples show differences in mitochondrial morphology, mtDNA copy number, and ETC protein levels in disease as compared to wild type samples. (A) Images of PAS-stained tissue showing tubule injury at 8 weeks, but no signs of disease at 4 weeks; and mitochondrial morphology (SDHA) demonstrating mitochondrial puncta as early as 4 weeks of age (white arrowheads), before histological signs of disease. (B-C) Quantification of SDHA-positive pixel area and SDHA MFI. (D) Quantification of mtDNA copy number in proximal tubule epithelial cells from 8-week-old mice. (E) Western blots showing effect of disease on...
expression of nuclear DNA-encoded (Complex I, II, III, and V) and mtDNA-encoded (Complex IV) murine ETC subunits in proximal tubule epithelial cells at 8 weeks of age. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, by 1-Way ANOVA with Dunnett’s post-hoc testing. n=4-10 per group.

on mtDNA copy number and ETC components in the proximal tubules of a mouse model of Alport nephropathy, renal PTECs were isolated from 129X1/SvJ background Col4a3^{+/+} mice at 8 weeks of age and from littermate Col4a3^{-/-} mice at 4 and 8 weeks of age. Changes in mtDNA copy number in 8-week-old mice were assessed in the isolated PTECs using DNA freshly collected from these cells. As shown in Figure 10D, there was a significant decrease in mitochondrial DNA copy number in Col4a3^{-/-} mice, as compared to wild type controls. Additionally changes in relative levels of the five complexes of the ETC were assessed using proteins collected from the freshly isolated PTECs. There were no significant changes in the expression of Complex I, II, or V (nuclear DNA encoded subunits), despite the observed aberrant mitochondria of Col4a3^{-/-} kidney samples by SDHA immunofluorescent staining (Figure 10A), as SDHA is a component of Complex II. In contrast, there were significant decreases in the expression of Complex III (a nuclear DNA encoded subunit) and Complex IV (a mtDNA encoded subunit). These are of interest since altered levels of assembly can arise from mutations in individual subunits or mutations in assembly factors for the complexes, which could have resulted from the increased cellular ROS levels known to be present in Col4a3^{-/-} mice at this age (Gomez et al., 2015). Altered levels of these complexes could also come as a result of mtDNA depletion, which is in agreement with the findings of Figure 10D.
Figure 11. CypD is effectively absent in Ppif/- mice and reduced in Ppif+/- mice, compared to Ppif+/+ mice. (A) CypD protein expression deletion in the kidney of 12-week-old mice was confirmed by western blotting. (B) Densitometric analysis of CypD normalized to β–Actin.

Genetic Ablation of CypD Expression Had No Effect on Weight Loss or Survival in Col4a3+/- Mice

To determine whether deletion of CypD (Ppif) has an effect in a mouse model of Alport nephropathy, a cross between the mixed C57BL/6 and 129 genetic background Ppif+/- mice and the 129X1/SvJ background Col4a3+/- mice was made (Table 3). Full or partial knockout of CypD was confirmed by western blot (Figure 11). Mice of the various genotypes were born in expected Mendelian ratios and appeared to be healthy, gaining weight normally until 7 weeks of age, the age of study enrollment. As expected, Ppif+/-;Col4a3+/- and Ppif+/-;Col4a3+/- mice were healthy and gained weight normally up to 16 weeks of age, the longest time point of data collection. In contrast, Ppif+/-;Col4a3-/- mice began to lose weight and showed signs of worsening body conditioning scores at 7 weeks of age as noted by segmentation of vertebral column, readily palpable dorsal pelvic bones, signs of dehydration (skin pinch), lethargy, abnormal locomotion, and/or ruffled fur. This same pattern was seen in Col4a3-/- mice regardless of the presence or absence of Ppif expression (i.e. In Ppif-/-, Ppif+/-, and Ppif+/-) (Figure 12, A and C).
Notably, significant mortality occurred in each of these three Col4a3<sup>–/–</sup> groups, with these mice requiring euthanasia as early as 8.5 weeks of age due to poor body conditioning scores and/or greater than 20% body weight loss. Figure 12C shows the age of euthanasia, if prior to age 16 wks, for each of the study groups. Thus, the average body weight changes over time shown in Figure 12A include only the surviving mice in each group at the given timepoint. As expected, there were no deaths in either the Ppif<sup>+/+</sup>;Col4a3<sup>+/+</sup> or Ppif<sup>–/–</sup>;Col4a3<sup>+/+</sup> groups, up through 16 weeks of age. The survival curve (Figure 12B) shows a significant difference between the two Col4a3<sup>+/+</sup> groups and the three Col4a3<sup>–/–</sup> groups, with no significant difference between the latter groups in the time to 50% survival (Ppif<sup>+/+</sup>;Col4a3<sup>–/–</sup>, 87 days), Ppif<sup>–/–</sup>;Col4a3<sup>–/–</sup>, 82 days), and Ppif<sup>–/–</sup>;Col4a3<sup>–/–</sup>, 84 days).

Full or Partial CypD Ablation Fails to Protect Col4a3<sup>–/–</sup> Mice From Loss of Kidney Function as Compared to Ppif<sup>+/+</sup>;Col4a3<sup>–/–</sup> Mice

To further understand the impact of CypD deletion on Alport kidney disease, renal function was evaluated in mice at 12 weeks of age in an observer-blinded manner. As noted in Figure 12, it was typical for some mice to die before 12 weeks of age, as the survival range for the Col4a3<sup>–/–</sup> mice was between 61 - 117 days (~8.7 - 16.7 weeks) of age. Thus, in this section, our investigations will only cover the survivors, which could overestimate the renal function in Alport cohorts.

To determine the renal function of these mice, analysis of blood urea nitrogen (BUN) and urine albumin/creatinine ratio was performed. In both assays, the results revealed that Col4a3<sup>–/–</sup> mice (with Ppif sufficiency, partial Ppif sufficiency, or Ppif
Figure 12. Full or partial Ppif deletion has no effect on life span or protecting Col4a3-/- mice from loss of kidney function. (A) Curve showing mean body weight changes (± SEM) with time up to 16 weeks of age. (B) Kaplan-Meier survival curve. (C) Individual weights for mice in survival study and age of euthanasia [indicated by x] if prior to 16 weeks of age. (D) Plasma BUN levels at 12 weeks. (E) Urine albumin concentration normalized to urine creatinine in timed urine collections at 12 weeks.
deficiency) had significantly decreased renal function as compared with Col4a3^{+/+} mice (with *Ppif* sufficiency and deficiency) (Figure 12, D-E). There were no significant differences within the Col4a3^{−/−} group (with *Ppif* sufficiency, partial *Ppif* sufficiency, or *Ppif* deficiency), indicating the course of the disease was not altered by CypD deletion at this time point.

Full or Partial CypD Ablation Fails To Reduce Renal Histopathology in Col4a3^{−/−} Mice as Compared to *Ppif^{+/+};Col4a3^{−/−}* Mice

To further understand the impact of CypD deletion on Alport kidney disease progression, histological parameters were evaluated in samples harvested at 12 weeks of age in an observer-blinded manner. As noted in Figure 12, it was typical for some mice to die before 12 weeks of age, as the survival range for the Col4a3^{−/−} mice was between 61 - 117 days (~8.7 - 16.7 weeks) of age. Thus, in the subsequent sections, our investigations will only cover the survivors, which could underestimate the pathology in Alport cohorts.

To determine whether mice displayed significant tubulointerstitial pathology typical of Alport nephropathy, tissue sections were analyzed for signs of tubular injury/atrophy (Figure 13, A-B) and interstitial fibrosis (Figure 14B and Figure 15). Quantification of these results revealed that Col4a3^{−/−} mice (with *Ppif* sufficiency, partial *Ppif* sufficiency, or *Ppif* deficiency) were significantly different than Col4a3^{+/+} mice.
Figure 13. Full or partial Ppif deletion does not prevent tubule injury, glomerulosclerosis, or glomerular crescents in Col4a3-/- mice. (A) PAS-stained images of kidney cortex from mice at 12 weeks. (B) Percent of tubules showing sings of tubular injury at 12 weeks. (C) Percentage of fields analyzed that contained signs of glomerular crescents at 12 weeks. (D) Percentage of fields analyzed that contained signs...
of global sclerosis at 12 weeks. (E) Percentage of fields analyzed that contained signs of focal segmental glomerulosclerosis at 12 weeks. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, by 1-Way ANOVA with Dunnett’s post-hoc testing. n=10-15 per group.

(with Ppif sufficiency and deficiency) in the following readouts: tubule injury score measured the percent of tubules showing signs of tubular injury (Figure 13B) and interstitial fibrosis (Figure 14B And Figure 15). When investigating for signs of tubule epithelial cell apoptosis, images from tissue sections stained for Cleaved Caspase-3 (Asp175) showed that nearly all tubules in Col4a3−/− mice stained positive (Figure 14A), regardless of the full or partial deletion of Ppif. As a negative control, Col4a3+/− sections were permeabilized and stained with secondary antibody only, thus demonstrating the specificity of the positive experimental staining results.

To determine whether mice displayed glomerular changes typical of Alport nephropathy, tissue sections were analyzed for signs of glomerular epithelial cell hyperplasia (glomerular crescents), glomerular fibrosis with capillary destruction (glomerulosclerosis), and periglomerular fibrosis (FSGS) (Figure 13A). Quantification of these results revealed that Col4a3−/− mice (with Ppif sufficiency, partial Ppif sufficiency, or Ppif deficiency) were significantly different than Col4a3+/+ mice (with Ppif sufficiency and deficiency) in the following readouts: glomerular crescents and global sclerosis (Figure 13, C-D). When comparing for signs of FSGS, however, only Ppif+/−;Col4a3−/− mice were significantly increased by this measure (Figure 13E).
Figure 14. Full or partial Ppif deletion does not prevent tubular apoptosis, interstitial fibrosis, myofibroblast expansion, leukocyte expansion, or endothelial rarefaction in Col4a3⁻/⁻ mice. (A) Images of tubular apoptosis (Cleaved Caspase-3) at 12 weeks, as compared to Col4a3⁻/⁻ stained only with secondary antibody (Negative Control). (B-E) Quantification of interstitial fibrosis (Sirius Red), myofibroblasts (αSMA), leukocytes (CD11b), and capillary density (CD31). ns = Not Significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, by 1-Way ANOVA with Dunnett’s post-hoc testing. n=8-15 per group.
CypD Ablation Failed to Reduce Inflammation/Myofibroblasts or Maintain Capillary Density In $Col4a3^{-/-}$ Mice

Molecular markers were used to assess effects on fibrogenesis, inflammation, and capillary density. No statistically significant changes were detected in expression of α-smooth muscle actin (αSMA) in $Col4a3^{-/-}$ mice (with $Ppif$ sufficiency, partial $Ppif$ sufficiency, or $Ppif$ deficiency) when compared with $Col4a3^{+/+}$ mice (with $Ppif$ sufficiency and deficiency) (Figure 14C and Figure 15), which is unusual for this model. However, $Col4a3^{-/-}$ mice (with $Ppif$ sufficiency, partial $Ppif$ sufficiency, or $Ppif$ deficiency) had significantly increased leukocyte infiltration, as measured by CD11b, when compared to $Col4a3^{+/+}$ mice (with $Ppif$ sufficiency and deficiency) (Figure 14D and Figure 15). Finally, $Col4a3^{-/-}$ mice (with $Ppif$ sufficiency, partial $Ppif$ sufficiency, or $Ppif$ deficiency) had significantly reduced capillary density, as measured by CD31, when compared to $Col4a3^{+/+}$ mice (with $Ppif$ sufficiency and deficiency) (Figure 14E).
Figure 15. Ppif deletion does not prevent interstitial fibrosis, myofibroblast expansion, or leukocyte expansion in Col4a3−/− mice. Representative images of collagen (Sirius Red), myofibroblasts (αSMA, and leukocytes (CD11b). g = Glomerulus; a = Arteriole.
Chapter IV.

Discussion

CKD is the ninth leading cause of death in the United States, threatening the lives of over 30 million CKD patients. Features of CKD include tubule injury, deposition of pathological ECM, and mitochondrial dysfunction – characteristics also found in CKD due to Alport nephropathy in man and mouse. The mPTP potentially contributes in this context by forming in response to elevated levels of intracellular calcium or ROS, thereby leading to the uncoupling of OxPhos and a loss of mitochondrial ATP synthesis. This loss of OxPhos disproportionately affects the renal proximal tubule and could lead to PTEC mitochondrial dysfunction and PTEC crosstalk with pericytes, who then detach from the capillary wall and transition into pro-fibrotic myofibroblasts. Since CypD has been shown to be a critical modulator of mPTP formation, CypD ablation was hypothesized here to prevent mPTP opening and thus reduce PTEC stress or death, thereby preventing or delaying the onset of fibrotic disease in a model of Alport nephropathy. This was supported by reports showing an improvement in human patients and animal models of Alport nephropathy elicited by CsA treatment, which blocks the activity of CypD. The studies described herein show that genetic ablation of CypD, as an approach to limit mPTP formation in a mouse model of Alport nephropathy, failed to improve renal function or histopathology and had no effect on the median life span of mice that had a relentless progressive kidney disease. As such, pharmacological targeting of CypD likely
does not represent a feasible approach for new therapeutics to treat Alport nephropathy patients.

In this study, we demonstrate alterations in the mitochondria of human CKD patients and a mouse model of Alport nephropathy. Within human samples of FSGS and DN, there is a significant difference in the staining pattern within the tubular epithelium of SDHA, a subunit of Complex II of the ETC found specifically within mitochondria (Figure 9A). As antibodies that recognize ETC complex subunits are used to visualize mitochondria in fixed samples, SDHA is considered a good proxy for determining mitochondrial morphology in these samples. While the human control samples appear to have a reticulum-like morphology, demonstrating a many-branched structure, both the FSGS and DN samples appear to have a fragmented morphology with many ovoid-shaped mitochondria. It should be noted, however, that it is possible for the cell to down-regulate the expression of these different ETC subunits, like SDHA, thus the staining pattern may reflective of this change in expression rather than a change in morphology. Yet, despite the decrease in mitochondrial area by SDHA immunofluorescent staining in diseased human kidney samples, there was a significant increase in the protein expression of the nuclear DNA-encoded ETC component SDHA, as well as COX10, in Stage 5 CKD PTECs (Figure 9, C-D). One possible explanation for this discrepancy is that the increased SDHA in PTECs could be an artifact of the PTEC isolation process and the only cells that survive this process are those that are extremely healthy and thus have an unusually high expression level of this protein. Another possible explanation is that this discrepancy may reflect a compensatory mechanism that most cells undertake to mitigate the loss of ETC function caused by mutated mtDNA or decreased mtDNA copy numbers.
For example, as noted above, the $Col4a3^{-/-}$ mice exhibit a similar alteration in SDHA staining pattern, and the PTECs from these mice had a significantly decreased mtDNA copy number without a decrease in the expression of Complex II, of which SDHA is a subunit (Figure 10, A-E). While mtDNA copy number was not quantified in the CKD human patient samples, a loss of mtDNA copy number might also underlie the trend towards a down-regulation of COX1, the mtDNA-encoded catalytic subunit necessary for the functioning of complex IV of the ETC, that was noted in Stage 5 CKD tissue (Figure 9, C-D). Additionally, the noted decreased mtDNA in the murine samples could also play a role in the decreased protein levels of the ETC subunits that are labile when their complexes are not assembled, as was seen with UQCRC2 of Complex IV (Figure 10E). Alternatively, as increased ROS can lead to mtDNA mutations in individual ETC subunits or mutations in assembly factors for the complexes, it is possible that the increased presence of ROS affected expression levels of COX1. This study thus makes it clear that mitochondrial alterations are present in both human and murine renal tissue in chronic renal disease.

Addressing the mitochondrial alterations associated with fibrosing kidney diseases has become an approach of increasing interest for treating CKD. In studies looking at biomarkers of oxidative stress present in the plasma of human CKD patients, evidence was found to suggest that oxidative stress increased as CKD progressed (Oberg et al., 2004). Furthermore, increased oxidant stress levels correlated significantly with the decline of renal function (Dounousi et al., 2006). In the $Col4a3^{-/-}$ mouse model of Alport nephropathy, mitochondrial dysfunction has been demonstrated by increased ROS present in the kidney tissue and urine. Additionally, these mice also show a significant
increase in renal tubule cell apoptosis and a significant decrease in the RNA and protein levels of Mpv17L (Gomez et al., 2015), a mitochondrial antioxidant restricted to PTECs in the kidney (Chau et al., 2012). MiR21, a small noncoding RNA, is increased in the renal tissue of human CKD patients and Col4a3−/− mice (Gomez et al., 2015) and is believed to contribute to the progression of CKD by promoting the formation of ROS (Zhang et al., 2012). One way that mir21 promotes ROS accumulation is by down-regulating TNFα, which then indirectly reduces expression levels of the ROS scavenger SOD2 (Zhang et al., 2012). This fact is of particular interest as our study demonstrates that expression of SOD2, one of the major enzymes metabolizing ROS, is decreased in Stage 5 human CKD patient PTECs (Figure 9, C-D). Notably, in a rat model of diabetic kidney disease, type 2 diabetic rats that were treated with Sitagliptin (a DPP-4 inhibitor shown to down-regulate miR-21 expression and promote the activation of the Nrf2 antioxidant pathway), treatment successfully reduced histopathological signs of disease (Civantos et al., 2017). Being that an increased presence of ROS has been linked to mPTP activation, it has been hypothesized that CKD patients may thus be susceptible to renal mitochondrial dysfunction associated with formation of the mPTP, which can lead to increased ROS production, ATP depletion, and cell stress/death (Zorov, Juhaszova, & Sollott, 2014). Furthermore, as the renal proximal tubule has a low capacity for glycolysis (Feldkamp et al., 2009), the mitochondrial dysfunction known to be present during CKD could potentially cause the proximal tubule to become particularly susceptible to cell stress and death following mPTP activation. Since signals associated with cell stress are believed to activate the differentiation of pericytes into myofibroblasts, the cells

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responsible for tissue fibrosis, mitochondrial dysfunction associated with activation of the mPTP could be a driver of chronic kidney fibrosis.

Inhibition of mPTP formation to address CKD is already under investigation, as a patent filed in 2012 seeks to find novel ways to modulate CypD biological activity as a means to treat a variety of kidney diseases (Padanilam, 2012). This therapeutic mechanism has been further supported by studies utilizing CsA (a known powerful inhibitor of CypD and thus also an inhibitor of mPTP formation) in human Alport nephropathy patients and animal models of Alport syndrome. In these studies, human patients showed a resolution of proteinuria for up to 10 years during continuous treatment of CsA (Callis et al., 1999). Additionally, a canine model of Alport syndrome revealed that treatment with CsA delayed progression of multilaminar changes to the glomerular basement membrane (Chen et al., 2003). However, as CsA is also a potent immunosuppressant, it was unclear what its major mechanism is for slowing disease progression. Furthermore, CsA is a known nephrotoxin (Calne et al., 1978). Thus the compound exerts both beneficial and toxic activities in the setting of kidney disease. This complexity may therefore be the cause for the limited and discordant data demonstrating efficacy of CsA in Alport syndrome (Charbit et al., 2007; Massella et al., 2010). Thus, the lack of consistent evidence could be misleading when considering using inhibition of CypD activity as a treatment for Alport nephropathy.

To date, only CypD has been agreed upon by numerous independent laboratories as being a critical regulator of Ca^{2+} and oxidative stress-induced mPTP opening, with substantial genetic and pharmacological studies having identified the role of CypD in potentiating pore opening (Baines et al., 2005; Basso et al., 2005; Hansson et al., 2011).
In the current study, full or partial genetic ablation of CypD resulted in no measurable changes, positive or negative, in disease outcomes in the Alport model of CKD: no difference in disease progression as measured by lifespan; no improvement in renal function; no decrease in tubular or glomerular pathology; no decrease in fibrosis, the number of myofibroblasts, or the infiltration of leukocytes; and no preservation of capillary density. However, as previous studies have shown that at very high levels of Ca\(^{2+}\), permeability transition can still be elicited in Ppi\(f^{-/-}\) mice (Baines et al., 2005; Millay et al., 2008; Ramachandran, Lebofsky, Baines, Lemasters, & Jaeschke, 2011; Wissing, Millay, Vuagniaux, & Molkentin, 2010), this suggests that while CypD may control the sensitivity of mPTP opening, alternative mechanisms of pore opening also exist under more extreme conditions, which could have played a role in our study.

Another possible limitation in our ability to detect a role for CypD is raised by the CypD protein levels observed in the kidney tissue in the various strains of mice in this study. Though CypD ablation successfully occurred in the context of CKD, as was shown by the western blot for CypD in the renal tissue from 12-week-old mice (Figure 11), the level of CypD protein normalized to \(\beta\)-Actin in Ppi\(f^{+/+};Col4a3^{+/+}\) mice was approximately six times greater than that of Ppi\(f^{+/+};Col4a3^{-/-}\) mice. This difference in expression of the mitochondrially-restricted protein CypD is likely due to the loss of mitochondria that occurs with disease progression, consistent with our observation of a significant decrease in mtDNA and a loss of SDHA\(^{+}\) staining area in the pure background strain Alport model (Figure 10). Alternatively, however, we cannot rule out that this reduced CypD expression in the Col4a3\(^{-/-}\) strains occurred at an early age, prior to disease onset, posing some limitation in the use of these strains to evaluate the hypothesis of
CypD-dependent mPTP formation in CKD. Given that the relative expression levels of CypD are less different between the three $Col4a3^{-/-}$ strains than is the case between the two $Col4a3^{+/+}$ strains, the functional contribution of CypD in CKD may be difficult to discern. To further dissect this result in future experiments, one could perform a time course measuring CypD expression in the Alport kidney, normalized to a mitochondrial loading control.

An additional factor that may have impacted the precision of the outcome of the present study is that we utilized $Ppif^{-/-}$ mice on a C57BL/6 and 129 mixed genetic background, crossing these with the $Col4a3^{+/+}$ mice on a 129X1/SvJ pure genetic background. Due to the mixed background of this novel cross, it was anticipated that the timing of disease onset, weight loss, and death might be different than the reported 76-day median survival of the 129X1/SvJ $Col4a3^{-/-}$ mice. Indeed, the median survival in this experiment was 82-87 days of age. Additionally, the range of survival was much broader in the mixed background strain (61-117 days) than in the pure background strain (60-85 days) (Gomez et al., 2015). Numerous studies have noted differing fibrotic responses in C57BL/6 mice when compared to 129 mice (Hartner, Cordasic, Klanke, Veelken, & Hilgers, 2003; Kato et al., 2008). In one example, $Col4a3^{-/-}$ mice on the 129X1/SvJ background were reported to reach end stage renal failure (ESRF) at ~66 days of age, while on a pure C57BL/6J background the mean age at ESRF was 194 days of age (Andrews, Mudd, Li, & Miner, 2002). To determine why this may be the case in the $Col4a3^{-/-}$ mice, Kang et al. investigated a strain-specific alternative collagen IV isoform switch in the glomerular basement membrane (GBM) that may lessen the disease severity. In their study, they found that the absence of $\alpha3/4$(IV) collagen chains in the
GBM in these mice was found to sometimes induce ectopic deposition of α5/6(IV) collagen. The GBM deposition of α5/6(IV) collagen was abundant in C57BL/6 Col4a3^−/− mice, but was almost undetectable in 129X1/Sv Col4a3^−/− mice. Importantly, high levels of ectopic α5/6(IV) collagen in the GBM were associated with approximately 46% longer renal survival (Kang et al., 2006). Thus, though we increased our ‘n’ in an attempt to avoid issues related to the mixed background strain utilized in this study, the known effects of the C57BL/6 background on CKD progression could explain why we saw such variation in our results, which made it difficult to discern whether there was any beneficial effect from Ppif deletion.

To support or refute the outcomes of the present study, additional experiments should be performed to determine whether inhibition of mPTP formation represents a promising therapeutic avenue for human CKD patients. Firstly, the investigation described herein should be repeated on a pure 129SvJ background strain to demonstrate whether genetic ablation of CypD expression can ameliorate progression of disease in a mouse model of Alport nephropathy. Paired with this, investigators that are able to develop a novel, specific small molecule inhibitor of CypD should test it using the Col4a3^−/− mice on the pure 129SvJ background strain, as studying Alport nephropathy in animals is advantageous since they lack the comorbid conditions often associated with other, older CKD populations and they always progress to ESRF. Finally, as the Alport model may not necessarily be indicative of all CKD, future investigators should induce CKD using the unilateral ureter obstruction or folic acid models to add support to the role of mPTP formation in many models of CKD. Furthermore, in addition to expanding the models used, one could also perform additional experiments to interpret the proximal and
distal outcomes of CypD deletion or inhibition in a disease setting. For example, one
could determine whether there are changes in disease progression in the Ppif⁺/⁺;Col4a3⁻/⁻
mice as compared to controls by collecting urine and blood plasma at serial timepoints to
form a time course of renal function. Furthermore, one could also determine the amount
of ATP present in Ppif⁺/⁺;Col4a3⁻/⁻ whole kidney tissue or in isolated PTECs as compared
to controls using the ENLITEN ATP Assay System (Promega), as has been previously
reported (Devalaraja-Narashimha et al., 2009). In so doing, one would expect that if
CypD deficiency successfully prevented mPTP formation in this model, there would be
significantly more ATP present in the kidney tissue and PTECs of Ppif⁺/⁺;Col4a3⁻/⁻ mice
as compared to Ppif⁺/+;Col4a3⁻/⁻ mice. Alternatively, one could investigate the
mitochondrial respiration effects of pharmacologic CypD inhibition in Col4a3⁻/⁻ PTECs
ex vivo in the presence of an in vitro mPTP inducer using the Seahorse Extracellular Flux
system (Agilent). By performing the proposed experiments described above, one can
definitively determine whether inhibition of CypD activity is sufficient to ameliorate
disease progression in an animal model of CKD.

In addition to testing CypD deficiency or pharmacological inhibition to validate a
therapeutic approach to CKD, further methods to inhibit mPTP formation should also be
tested. As described above, though CypD is known to be a critical regulator of Ca²⁺ and
oxidative stress-induced mPTP opening, high levels of intracellular Ca²⁺ still allow for
mPTP opening to occur, even in the presence of CypD inhibitors or with Ppif deletion.
One such alternative pharmacological inhibitor is ER-000444793, which has been shown
to be a CypD-independent inhibitor of mPTP in vitro. As compensatory mechanisms may
exist to allow for mPTP activation to still occur under conditions of extreme stress, such
as may be present in CKD, using an inhibitor with a CypD-independent mechanism could be therapeutically beneficial in this context.

To conclude, genetic deletion of CypD has not been shown in this study to represent an effective approach for retarding the downstream consequences of mutations in type IV collagen genes that ultimately drive progression of Alport nephropathy. However, such an approach may potentially inhibit progression of other forms of CKD and may yet prove useful in Alport nephropathy in a mouse model on a pure background strain. Alternatively, pharmacologic inhibition of mPTP formation independent of CypD could have a more robust effect in these models and is another avenue worth pursuing. As genetic ablation of CypD expression has already been shown to be protective in renal models of ischemia reperfusion injury, as well as chronic disease models in other organs, inhibiting mPTP formation remains an attractive target for addressing human disease.


