Genetic Dissection and *In Vivo* Modeling of Sickle Cell Disease Nephropathy

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program in Genetics and Genomics
in the Graduate School
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ABSTRACT

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Abstract

A common complication among sickle cell disease (SCD) patients is the development of renal disease. Paradoxically, the incidence of chronic kidney disease (CKD) increases as patient survival improves, and as such the development of sickle cell disease nephropathy (SCDN) has become an emergent health concern in SCD. For individuals with sickle cell anemia (SCA), albuminuria rates are as high as 68% in adult patients and as many as 18% of these patients progress to end-stage renal disease (ESRD). The detection of SCDN relies on relatively late markers of the disease process, namely proteinuria and reduced glomerular filtration rate (GFR), delaying identification of at-risk SCD patients prior to organ damage. Thus, early detection of those at risk is required to reduce morbidity and mortality among SCD patients.

In order to accomplish this, we have used a tiered approach employing genetic association strategy in patient populations and functional examination in relevant zebrafish model systems. We demonstrated previously that *MYH9* and *APOL1*, in linkage disequilibrium on chromosome 22, are strong, independent predictors of risk for proteinuria in SCD. This region, particularly two major risk variants (named G1 and G2) in *APOL1*, has been replicated widely in non-SCD nephropathy and represents one of the strongest genetic signals for a complex human phenotype. Using the zebrafish system, we discovered a functional role for *APOL1* in the developing zebrafish kidney

and uncovered a complex genetic architecture, in which the G2 allele exerts adverse functions on the kidney and kidney cell types. Critically, we also found that *APOL1* and *MYH9* interact genetically, particularly in the context of anemic stress, which we also observed in a SCD patient population.

However, variants at the *MYH9*/*APOL1* locus appear to only explain a part of the disease risk, suggesting that additional genetic factors may be contributing to renal outcome in SCD patients. As such, we performed an unbiased interrogation of the genome (GWAS) in order to uncover putative new nephropathy genes for genetic evaluation. Using a host of genetic methods to identify both common and rare variation present in SCDN individuals, we identified seven candidate loci associated with renal outcome in SCD. Again, using zebrafish models, we provide relevant functional evidence for a subset of these genetic candidates by assessing their effect on glomerular filtration barrier integrity.

Collectively, these genes and markers may indicate novel genetic mechanisms contributing to SCD nephropathy, and may further refine diagnostic paradigms for identifying those patients most at risk. In addition, these results stand to make significant progress in identifying novel therapeutics for SCDN.

Dedication

To my parents, Greg and Joyce Anderson, who gave me the skills to get this far and cheered me on in all of my intellectual pursuits. They are the best of parents who made me believe I really can do anything. To my brother and sister, Brad and Stephanie Anderson, who always support and inspire me. To my wife, Jennifer Anderson, and my daughter, Marie Anderson, who have been an endless source of love through graduate school and everything else I do. You both are the light of my life and are the reason I have been able to get this far.

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1. The Genetic Basis and Clinical Outcomes of Patients with Sickle Cell Disease

1.1 Genetic basis of sickle cell disease

Sickle cell disease (SCD) was the first human disease to be molecularly characterized, which was identified by Linus Pauling in 1949[1] and, in the same year, shown to have an autosomal recessive form of inheritance.[2] In later years, Ingram and colleagues further characterized the disorder, describing SCD to contain a single glutamine-to-valine substitution at the sixth position in the β-globin peptide (HbS).[3] Over the next few decades, the organization of the human globin proteins was further determined and we now know that the hemoglobin molecule is actually a tetramer, consisting of two pairs of identical peptide subunits. These subunits are encoded by different sets of genes and are arranged in the same order in which they are expressed

during human development. The α-globin and β-globin genes are located on chromosome 16 and chromosome 11, respectively, and together are temporally expressed during fetal life and on into adulthood (Figure 1). In fetal stages, the predominant type of hemoglobin present is HbF ($\alpha_2\gamma_2$) and this is gradually replaced by HbA ($\alpha_2\beta_2$) in postnatal development. To accomplish this sensitive expression timing, each gene set is under the control of a major regulatory element (β-LCR for the β-globin genes and HS-40 for the α-globin genes) located far upstream of the genes in the cluster. These regulatory elements are necessary for the high level of expression of either the α- or β-globin proteins. It is during this developmental switch from expression of HbF to HbA in which the clinical features of SCD begin to manifest in patients with mutated forms of the β-globin genes.

Although SCD is often described as a disease caused by a single point mutation, it actually consists of a group of disorders, all of which occur when an individual inherits two abnormal copies of the β-globin gene. Homozygosity for the sickle mutation (HbSS) results in the most common and most severe form of SCD, sickle cell anemia (SCA). However, several other mutations in the β-globin gene, when they are co-inherited with one copy of the HbS mutation, can cause SCD with various clinical severity (Table 1). HbSC ($\beta^S\beta^C$) disease is usually a very mild form of SCD resulting in less severe anemia and pain crisis compared to HbSS SCD patients.[4] β-thalassemia

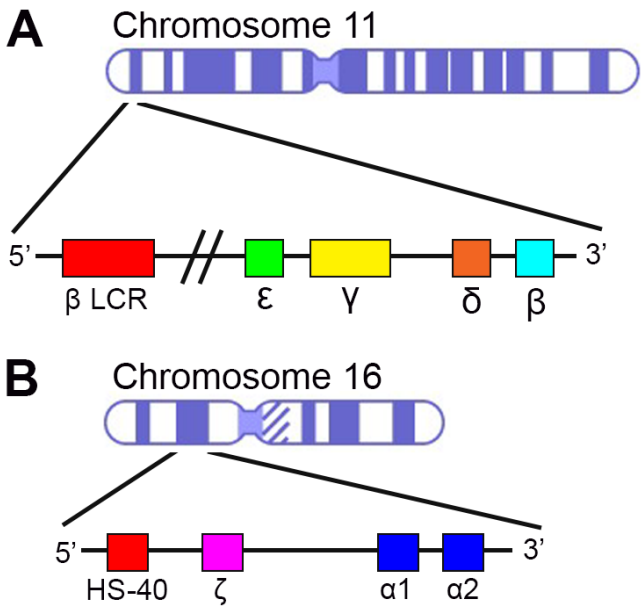

Figure 1: Genome organization of the α- and β-globin gene clusters.

(A) The β-globin gene cluster is located on chromosome 11. Each gene is expressed in the same order in which it is present in the cluster. During fetal growth and shortly after birth, the predominant type of hemoglobin present is HbF ($\alpha_2\gamma_2$). Hemoglobin switching is controlled by the β-locus control region (β-LCR) and leads to the silencing of γ-globin and the activation of adult β-globin expression, replacing HbF by HbA ($\alpha_2\beta_2$). (B) The genes of the α-globin cluster are located on chromosome 16. HS-40 is the major regulatory element that results in the high level of expression of the α-genes.

Table 1: Genotype and severity of sickle cell disorders

Hb Type	Genetic Interaction	Clinical Severity*
HbAA	β and β	None
HbSS	β^S and β^S	Severe
HbSC	β^S and β^C	Mild
HbSβ^0	β^S and β^0-thalassemia	Severe
HbSβ^+	β^S and β^+-thalassemia (severe)	Moderate
HbSβ^+	β^S and β^+-thalassemia (mild)	Mild

*This refers to the most common clinical presentation. In some cases, the genotype may not predict the clinical severity of the disease.

mutations ($Hb\beta^0$ or $Hb\beta^+$) vary in the degree of SCD severity when coinherited with an HbS mutation. If the β-thalassemia mutation causes complete inactivation of the beta-globin gene ($Hb\beta^0$) the severity of disease typically is similar to SCA.[5] However, when the β-thalassemia mutation results in partial inactivation of the gene ($Hb\beta^+$), the clinical severity of SCD can be highly variable. Interestingly, the severity of SCD seen in $Hb\beta^+$ patients seems to depend on the population substructure, as people of African descent with a $Hb\beta^+$ mutation tend to have milder symptoms compared with Mediterranean populations with a $Hb\beta^+$ mutation.[6] This suggests there may be population-specific genetic modifiers at other genetic loci. In contrast to homozygous deleterious mutations in β-globin, heterozygous carriers of HbS do not incur any symptoms of SCD. Only in rare and extreme cases do individuals with sickle cell trait (SCT; HbAS) experience complications, such as pain crisis. These can occur during conditions of increased atmospheric pressure or low air oxygen levels.

The primary pathophysiological response to homozygous mutations in β-globin is the formation of intracellular hemoglobin (Hb) polymers under conditions of deoxygenation. These Hb polymers are induced when the mutant β chain binds to a complementary hydrophobic site (as a result of the valine replacement in the protein) and the formation of multiple polymers within a red blood cell (RBC) cause conformational cytoskeleton changes which give rise to its sickled appearance. This drastically reduces the elasticity of RBCs and can cause the sickled cells to adhere to

each other, occluding small blood vessels throughout the body and inducing vascular injury.[7] Sickled RBCs also have an increased propensity (compared to normal RBCs) to adhere to vascular endothelial cells, resulting in further vasoocclusion and tissue damage.[8, 9] In addition, sickled RBCs become dehydrated, which in turn causes further deoxygenation and so exacerbates sickling and RBC aggregation. It has also been shown that HbSS RBCs have decreased nitric oxide, adenosine triphosphate content and antioxidant capacity, which can cause extensive oxidative damage to many cellular components and lead to intracellular abnormalities.[10-13] Finally, the increased fragility of HbSS RBCs (due to their sickled nature) results in extensive hemolysis and anemia in SCD, causing fatigue and low oxygen retention in patients.[14]

1.2 Clinical outcome and treatment in sickle cell disease

1.2.1 Clinical complication and survival in SCD

Contrary to the relatively simple genetic basis of SCD (Mendelian inheritance of a deleterious mutation in the β-globin gene), clinical manifestations are diverse and complex across the patient population. As such, SCD prognosis is highly variable between individuals. Despite its complex presentation, however, overall survival of SCD patients has drastically improved over the last 40 years. In the U.S. in the 1970s the median survival of HbSS SCD patients was only 14.3 years.[15] Through the development of clinical management improvements over the last few decades, however, the median survival in an adult contemporary SCD patient cohort was found to be 60 years of age.[16]

Thus, most SCD patients are now living well into adulthood. Still, much research is focused on the identification of clinical outcomes and phenotypes that predict unfavorable prognosis in the SCD population. Studies have shown that renal failure, stroke, acute chest syndrome (ACS), low fetal Hb levels, frequent pain crisis, cardiopulmonary complications, and baseline white blood cell count (WBC) to be associated with overall decreased survival in SCD patients.[4, 16, 17] Molecular markers have also been associated with survival in SCD, such as adhesion molecules (VCAM-1 and E-selectin) and inflammatory proteins (IL-6, IL-8 and IL-10).[4, 18, 19] Despite much progress, however, SCD complications still inhibit quality of life and result in an overall shortened lifespan compared to the general population. The identification of molecular or genetic markers predicting risk for some of the most dire outcomes in SCD populations could greatly assist in prognosis and clinical intervention in patients.

1.2.2 Treatment options in SCD

There is currently no known cure for sickle cell disease, although treatment options for patients are constantly evolving. The closest curative care for SCD involves bone marrow transplantation, therein replacing the hematopoietic source of the defective sickle cells. However, this approach has long been deemed too risky as the procedure itself resulted in a roughly 20% mortality rate.[20] However, recent developments in immunosuppressive therapy and post-procedure care have prompted the use of bone marrow transplantation in clinical trials, showing a greater than 90%

long-term survival rate.[20-22] Despite these advances, very few transplantation procedures have been performed in adults with SCD due to morbidity and mortality risks.

Transfusion therapy has seen some success as an effective treatment for clinical outcome in SCD. Clinical trials of patients undergoing repeated transfusions show a reduction in the risk of recurrent stroke in children with SCA.[23] This rapid exchange of hemoglobin drastically reduces the concentration of HbSS to less than 30% for up to five years. However, repeated transfusions at this interval is not innocuous, as some studies show that up to 30% of patients with SCA who receive blood transfusions become alloimmunized.[23, 24] Furthermore, frequent transfusions increase the risk of infection and iron accumulates in several vital organs.[25] These hazards often limit transfusion treatment to those patients who have severe anemia or who have had a recent acute stroke.

Hydroxyurea (HU) is the only FDA approved drug for the treatment of SCD and is routinely orally administered in patients. Before its use in SCD, it had been used for many years for the treatment of myeloproliferative disorders as it inhibits ribonuclease reductase.[26] Studies have shown that treatment with HU markedly decreases pain crisis, acute chest syndrome, transfusion requirements, and overall hospitalizations in adults with SCD.[27] Additionally, over a nine year follow-up study, SCD patients treated with HU were shown to have an increase in survival.[28] Despite its wide use, however, we still do not have a clear understanding of its mechanism of action. We do know that HU

stimulates high HbF induction in SCD patients, which reduces the formation of HbSS polymers and therefore reduces the overall severity of SCD.[27, 29-31] More recent studies have shown that HU increases nitric oxide production *in vivo*, which upregulates γ-globin expression.[32] Yet another study points to the disruption of transcription factors by HU to bind to enhancer regions around the globin genes, and therefore alter the ratio of HbS to HbF.[33] It may also have benefits outside of increasing HbF, such as decreasing the adhesion of sickled RBCs to the endothelium and increasing the time of HbSS polymerization.[34, 35] The risks of treatment of SCD with HU are still unclear. Studies show a decrease in white blood cell count and platelets in SCD patients under HU treatment, and, in rare cases, it can worsen anemia.[27] However, these adverse effects seem to reduce drastically after omission for a period of time. Long term effects of HU treatment for SCD patients are widely unknown. Studies so far show no apparent increase in risk of cancers nor growth inhibition in children, but as widespread treatment only began in the 1990s, more studies are needed.

1.3 Renal manifestations in sickle cell disease

1.3.1 Introduction

One of the most severe and dire clinical outcomes in SCD is the development of renal disease. Paradoxically, the incidence of chronic kidney disease (CKD) increases as patient survival improves, and as such the development of sickle cell disease nephropathy (SCDN) has become an emergent health concern in SCD. For individuals

with SCA (HbSS disease), albuminuria rates are as high as 68% in adult patients and as many as 18% of these patients progress to end-stage renal disease (ESRD).[4, 36, 37] The onset of CKD is insidious and requires constant clinical care and monitoring to detect those patients who are most at risk for developing significant renal complications. Much research into the identification of risk factors that predispose patients to SCDN is needed to identify the subset of individuals at greatest risk for nephropathy.

1.3.2 The pathophysiology of SCDN

In the early stages of life, SCD patients display renal enlargement, although kidney function is normal.[38] The reasons for this are still unknown, but it has been postulated that this is due to the relative increase in hypoxia and acidosis present in the kidneys compared to other tissues, which favor sickling of HbS and cause vasodilation.[39] In contrast to other forms of CKD, SCD patients in the early stages of disease actually display hyperfiltration of blood through the kidneys, possibly as a result of the dilation of medullary capillaries due to sickling RBCs.[40, 41] This in itself causes many complications for earlier diagnosis of SCDN, as reduced glomerular filtration rate (GFR) is used as a clinical hallmark for kidney decline. However, hyperfiltration alone is not known to cause pathological damage, but when this is coupled with glomerular hypertrophy, it can lead to glomerular lesions and eventually sclerosis. In fact, renal histological findings in SCDN often resemble other forms of CKD, such as focal segmental glomerulosclerosis (FSGS) and membraneoproliferative glomerulonephritis.

Mesangial proliferation, thickening or reduplication of the glomerular basement membrane, fibrosis, and the presence of microvillus protrusions have also been reported in SCDN patients.[42-45]

Proteinuria is a common occurrence in SCD and progresses in an age-dependent manner. In the first three decades, macroalbuminuria (>300 mg/g creatinine) or microalbuminuria (30-300 mg/g creatinine) occur in up to 27% of SCD patients and in older patients as many as 68% display proteinuria.[39, 46] Additionally, in a five year follow-up study, the prevalence of CKD in SCD patients with albuminuria rose from 29% to 42% and the number of patients with CKD in stages 1, 2, 4 and 5 increased as well.[47] Glomerular permselectivity defects may be the cause of this drastic increase in proteinuria seen in SCDN, as studies have shown that the radius of restrictive pores in the glomerular filtration barrier are increased in patients.[48, 49] Given the high prevalence and pathophysiological findings of proteinuria in SCDN, it is often indicated as one of the more static predictors of kidney decline in SCD patients.

Endothelial dysfunction, concentrated in the glomerular capillaries and surrounding kidney tissue, is also evident in SCDN.[50, 51] Many of the clinical manifestations of SCDN are thought to derive from poor maintenance of the glomerular endothelium, and this integrity is dependent upon podocyte-derived signals.[52] Podocyte effacement or injury is central to the etiology of nearly all forms of CKD and SCDN pathophysiology appears to be no different. The interplay between podocytes and the

neighboring endothelium is crucial for forming proper slit diaphragms so the glomerular filtration barrier can function. Loss of podocyte integrity can cause reduced endothelial nitric oxide production, thereby inducing a proinflammatory and procoagulant response in the glomerular capillaries.[53, 54] In turn, endothelial dysfunction can then result in the release of inflammatory mediators (ET-1 and TNF) and engage receptors on podocytes, causing injury and inducing proteinuria.[54]

The increase in renal blood flow in SCD patients can often cause tubular abnormalities that result in kidney complications, especially later in life. Hyposthenuria (the inability to concentrate urine) is almost universally present in people with SCD and can cause severe dehydration.[55] In SCDN, hyperfiltration in the glomerulus causes enhanced clearance of the insterstitial solutes which severely limits the concentrating ability of the collecting ducts.[56] Increased GFR rate also deliver increased amounts of salt to the tubular filtrate, which further increases the hypoxic environment and can predispose many patients to tubulointerstitial injury.[57] The distal tubule is also often impaired in SCDN, which reduces potassium and hydrogen ion excretion, which can lead to renal tubular acidosis in patients. However, this is often secondary to other tubular abnormalities.[58-60]

1.3.3 Treatment for sickle cell disease nephropathy

The assessment of treatment options for SCD patients who develop nephropathy is plagued by the lack of large, long term controlled studies. Some small scale studies

have focused on the treatment of albuminuria in SCD with angiotensin converting enzyme inhibitors (enalapril and captopril).[61, 62] Treatment with either drug has shown promising results and reduces urinary protein excretion of almost all patients. However, no studies have been done to show if this effect actually decreases progression to CKD or halts SCD glomerulopathy. Furthermore, the use of angiotensin converting enzyme inhibitors has been shown to increase the rate of acidosis in other forms of CKD, and so their use presents a substantial risk in SCD patients.

Although hydroxyurea has been shown to be beneficial for treating many other clinical complications in SCD, its use for treating SCDN has not been assessed in a controlled setting. Some reports suggest reduction of albuminuria after treating with HU, and it may also slow the development of hyperfiltration in SCD.[63-65] However, although it may decrease proteinuria in patients, its effect on microalbuminuria is minimal.[66] HU appears promising, but larger studies will be needed to assess its efficacy in SCDN.

Renal replacement therapies and renal transplantation has been shown to improve survival in SCD patients, especially for those who receive pre-dialysis nephrology care early on in the disease progression.[67] However, due to the underlying risk of vaso-occlusion and cardiovascular crisis in SCD patients, transplantation recipients are at a much higher risk of thrombosis compared to other forms of CKD.[68, 69]

In addition, recurrent disease has been shown to occur in SCD patients after only 3.5 years post-transplant.[68]

1.4 Summary of clinical outcomes in SCD and the need for early identification of risk factors

Sickle cell disease is one of the most frequently inherited molecular diseases in the entire world and in its most common and severe form (HbSS), causes diverse and dire clinical outcomes for patients. Renal manifestations in SCD are especially calamitous and contribute substantially to reduced life expectancy, resulting in 16%-18% mortality among patients.[16] Paradoxically, as clinical care for SCD patients improves, SCDN occurrence actually increases and presents a growing burden on individual and health system costs.

The severity of many forms of SCD have been found to depend on the population substructure of patients, therefore suggesting that population-specific genetic modifiers may be useful predictors of clinical outcome risk.[5, 16] Especially in the case of SCDN, early detection of those at risk are required to reduce morbidity and mortality. Thus, further research into the identification of genetic modifiers contributing to renal outcome in SCD patients is of paramount importance.

2. Genetic Susceptibility to Sickle Cell Nephropathy

Parts of this chapter are adapted from the Introduction section of the published manuscript:

Anderson, B.R., Howell, D.N., Soldano, K., Garrett, M.E., Katsanis, N., Telen, M.J., Davis, E.E., and Ashley-Koch, A.E. *In Vivo* Modeling Implicates APOL1 in Nephropathy: Evidence for Dominant Negative Effects and Epistasis under Anemic Stress. PLoS Genetics 11(7): e1005349. doi:10.1371/journal.pgen.1005349 (2015)

2.1 Chronic kidney disease and genetic susceptibility in African Americans: MYH9 and APOL1

Compared to European Americans, African Americans have a disproportionate risk for several forms of chronic kidney disease (CKD), including human immunodeficiency virus (HIV)-associated nephropathy (HIVAN),[70] focal segmental glomerulosclerosis (FSGS),[71] hypertension-attributed CKD,[72] lupus nephritis,[73] and diabetic nephropathy.[74] This increased risk for CKD among African Americans may be partially explained by differences in socio-economic factors, lifestyle factors and clinical features (blood pressure control and hypertension)[75-77], but this increased incidence is retained even after these factors are taken into account.[78] As such, with the advent of genetic mapping and association studies, many groups began attempting to identify susceptibility genes for CKD and end-stage renal disease (ESRD) among African American populations.

Initial studies used mapping by admixture linkage disequilibrium (MALD), genome-wide method for identifying loci associated with diseases that have large ancestry-driven or racial disparities.[79] In a cohort of HIV-1-associated FSGS and idiopathic FSGS African American patients, a single genome-wide significant peak (MALD lod score = 12.4) located on chromosome 22 was found to be associated with the development of FSGS.[80] Further fine-mapping identified nine SNPs located within the gene non-muscle myosin heavy chain IIA, or *MYH9*, strongly associated with FSGS (OR = 1.76-2.96; $0.0004 \geq p \geq 1 \times 10^{-7}$).[80] In this same study, *MYH9* was also found to be

significantly associated with hypertensive ESRD among African Americans, but not with type 2 diabetic ESRD.[80] Published simultaneously, a study using MALD analysis in nondiabetic ESRD African American patients identified a genome-wide significant peak in the same region on chromosome 22 (22q12; MALD lod score = 8.56).[81] Again, upon further fine-mapping, SNPs in *MYH9* were determined to account for this strong association (OR = 1.17-3.10; $3.64 \times 10^{-3} \geq p \geq 3.63 \times 10^{-15}$).[81] Subsequent to these studies, a candidate genetic association study was performed in a cohort of type 2 diabetic ESRD (T2-ESRD) African American patients, which included 14 SNPs spanning the majority of the *MYH9* coding regions.[82] Contrary to association with non-diabetic nephropathy, *MYH9* SNPs were only found to be nominally associated with T2-ESRD (OR = 1.04-1.38; $0.0449 \geq p \geq 0.0381$).[82] Furthermore, this association was not replicated when expanded to include individuals from the Family Investigation of Nephropathy and Diabetes (FIND) cohort, suggesting that *MYH9*-nephropathy association may be restricted to non-diabetic forms of CKD.[82] Finally, in a cohort of unrelated African Americans with hypertension-associated ESRD (H-ESRD), 15 *MYH9* SNPs were genotyped that had been previously associated with FSGS and HIVAN.[83] *MYH9* SNPs were again found to be significant, with 71.8% of cases and 57.3% of controls homozygous for the risk haplotype (OR = 2.38; $p=1.22 \times 10^{-15-}$).[83]

Due to independent associations of *MYH9* SNPs with multiple forms of CKD in African Americans and its high frequency in this specific population, it seemed highly

plausible that this gene was a major susceptibility locus for kidney disease disparity in African ancestry populations. The current biological annotation also aligned with this hypothesis. *MYH9* is highly conserved among many mammalian species and is very similar to other non-muscle myosin forms.[84] Like muscle myosins, it binds to actin to perform various intracellular motor functions, including polarity maintenance and cellular trafficking.[85, 86] Prior to studies in CKD, mutations in *MYH9* had been associated with several autosomal dominant clinical syndromes, including May-Hegglin, Sebastian, Fechtner and Epstein.[87] All of these syndromes display macrothrombocytopenia, with variable additional pathologies, including deafness, cataracts, neutrophil Döhle-like bodies and, notably, glomerular disease.[87] MYH9 protein is found to be highly expressed in the kidney, liver, and platelets; and, within the kidney, expression has been specifically localized to the podocytes, glomerular capillaries, and nephric tubules.[88] Furthermore, mutations affecting podocyte proteins that also interact with the actin cytoskeleton, including CD2-associated protein[89] and synaptopodin[90], have been associated with FSGS in humans and experimental animal models, which suggests that maintenance of podocyte cytoskeleton is essential to ensure proper glomerular integrity and filtration functions. [89] Given its strong association and biological evidence, it was logical to presume that *MYH9* should be the primary candidate for risk assessment and therapeutic intervention in African American patients with CKD.

However, despite initial findings implicating *MYH9* in African American CKD, further fine-mapping studies were not able to locate causal or coding variants within *MYH9* responsible for the admixture signal.[91] In fact, the strongest SNP associations with idiopathic FSGS, HIVAN, and H-ESRD reside in introns 13, 14, and 15 of *MYH9*.[91] Two resequencing efforts only showed sporadic coding sequence variation, detecting no variation frequent enough to explain the observed *MYH9* associated haplotype.[91] These results led researchers to conclude that either: a) regulatory and/or splicing variation within *MYH9* may be responsible for African ancestry CKD risk, although the evidence for this is sparse, or (b) the "true" association may reside in a larger genomic interval than originally thought. Pursing this latter hypothesis, Genovese and colleagues[92] began investigating a larger genomic region that showed a strong signal of natural selection based on integrated haplotype score (iHS) data. Although this region included *MYH9*, it also spanned about 1 Mb upstream, which contained several apolipoprotein (*APOL*) genes. Using the newly available 1000 Genomes Project dataset, Genovese *et al.* performed an association analysis in a cohort of non-familial African American FSGS patients within this larger interval. Surprisingly, the strongest association found did not lie in *MYH9*, but instead in a 10-kb region in the last exon of apolipoprotein L-1 (*APOL1*).[92] The strongest signal was seen in a two-locus allele, which they termed G1, consisting of two nonsynonymous coding variants (S342G; I384M) in perfect LD ($p=1.07 \times 10^{-23}$).[92] After using logistic regression to control for G1, they identified a second signal

within *APOL1* that consisted of a 6-base pair deletion (N388_Y389del), termed G2, in close proximity to G1 ($p=4.38 \times 10^{-7}$).[92] Furthermore, when they controlled for the previously identified risk haplotypes in *MYH9*, the association in *APOL1* remained highly significant. Conversely, when they controlled for *APOL1* variants G1 and G2, no residual association in *MYH9* remained.[92] LD patterns in this region also show that G1 and G2 are in high LD with *MYH9*, with 89% of haplotypes carrying G1 and 76% of haplotypes carrying G2 present with *MYH9* risk variants.[92] Finally, when *APOL1* variants were tested in an extended cohort of H-ESRD African American patients, G1 and G2 were the strongest predictors of renal disease.[92] These results seemed to point to the *APOL1* G1 and G2 alleles as the "true" cause of the association in the 22q12 region with ESRD in African Americans.

It had been previously shown that *APOL1* confers resistance to the *Trypanosoma brucei brucei (T. b. brucei)* parasite through interaction with the lysosome.[93, 94] Two subspecies of *T. b. brucei* (*T. b. rhodesiense* and *T. b. gambiense*) have evolved the ability to infect humans through inhibition of *APOL1* trypanolytic activity by an acquired serum resistance-associated protein (SRA).[94, 95] As these parasites only exist in sub-Saharan Africa, and the fact that *APOL1* G1 and G2 variants have risen in frequency in African ancestry populations, but not in Europe or Asia, Genovese *et al.* hypothesized that *APOL1* may have undergone selective pressure in response to these trypanosome adaptations.[92] Using both human plasma and recombinant APOL1 proteins, they

showed that human APOL1 G1 or G2 variants were able to lyse the *T. b. rhodesiense* parasite, providing a possible biological explanation for this rise in natural selection at the locus (Figure 2).[92]

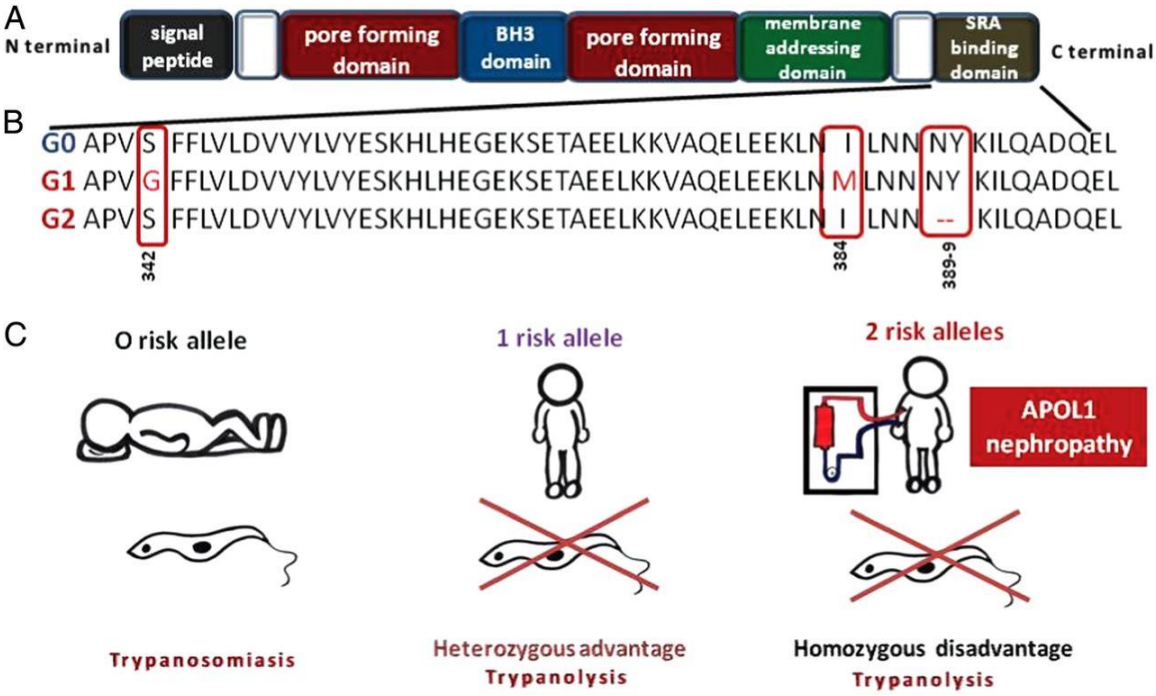

Figure 2: APOL1 domain structure and consequence of risk allele carriers.

(A) The structure of APOL1 with annotated domains. (B) G1 (S342G and I384M) and G2 (deletion of N388 and Y389) mutations are located at the C terminus. The close proximity of the G1 and G2 alleles results in a low likelihood of recombination between the two alleles. G2 is mutually exclusive from G1, and these two alleles never appear together on the same chromosome. Therefore, the two risk alleles include the following combinations: G1/G1, G1/G2 and G2/G2. (C) Illustration of the different consequences of APOL1 carriers: individuals with two APOL1 wild-type alleles (G0) are prone to T. b. rhodesiense infection. The depiction on the left represents an individual with zero risk alleles, suffering from African sleeping sickness. Individuals with one APOL1 risk allele (G1 or G2), represented by the depiction in the middle, are protected from T. b. rhodesiense infection and are likely at a lower risk for the development of APOL1-associated nephropathy. Individuals with two APOL1 risk alleles, represented by the depiction in the right are protected from T. b. rhodesiense infection but are at an increased risk for the development of progressive adult CKD. Figure is reprinted with permission, ©Kruzel-Davila, E., *et al.* 2015. Originally published in *Nephrol Dial Transplant*. 31(3):349-58.

Given the findings by Genovese *et al.* regarding this new insight into the association of the 22q12 region with African American disparity in CKD, many of the original groups that reported association with *MYH9* began investigating the frequency distribution of *APOL1* G1 and G2 alleles in their cohorts. Almost exclusively, G1 and G2 alleles in *APOL1* seemed to account for the "true" 22q12 association in all forms of African American CKD that were originally reported. Table 2 presents a full summary of *APOL1* associations with specific kidney diseases to date.

Table 2: Summary of *APOL1* risk alleles associated with kidney disease among African Americans.

Abbreviations: CI: confidence interval; ESRD: end-stage renal disease; FSGS: focal segmental glomerulosclerosis; HIVAN: HIV-associated nephropathy; H-ESRD: hypertension-associated end-stage renal disease

	N of patients	Odds Ratio (95% CI)	Percent of patients with 2 risk alleles	Reference
ESRD	430	6.7	37	Tzur *et al.* 2010
HIV-associated nephropathy	54	29 (13-68.5)	72	Kopp *et al.* 2011
Primary idopathic FSGS	217	17 (11-26)	72	Kopp *et al.* 2011
Combined FSGS/HIVAN	65	10.9	68	Papeta *et al.* 2011
Dallas Heart Study – kidney disease risk factor analysis	1825	3.9 (1.9-7.9)	13	Friedman *et al.* 2011
Case-only HIV+ biopsies	60 (HIVAN)	-	62	Atta *et al.* 2012
	35 (FSGS)	-	63	Fine *et al.* 2012
CKD and ESRD	304	3.89	31	Foster *et al.* 2013
Lupus glomerulopathy	26	5.4 (0.4-12.1)	50	Larsen *et al.* 2013
H-ESRD	675	2.6 (1.8-3.6)	23	Lipkowitz *et al.* 2013
Lupus ESRD	855	2.7 (1.8-4.2)	25	Freedman *et al.* 2014

2.2 APOL1 and MYH9 are both associated with sickle cell disease nephropathy

2.2.1 Introduction

The following section was adapted with permission from the published manuscript: Ashley-Koch, AE, Okocha, EC, Garrett, ME, Soldano, K, De Castro, LM, Jonassaint, JC, Orringer, EP, Eckman, JR, Telen, MJ: MYH9 and APOL1 are both associated with sickle cell disease nephropathy. British journal of haematology, 155: 386-394 (2011). *Although I was not part of this study, it is included in this section as it serves as a foundation for a substantial part of my dissertation research. Furthermore, the patient data set described in this study is the same that is used in novel analyses in later chapters. I have rewritten parts of the text for summary purposes, mainly the introduction and discussion sections.*

The recent findings into African American CKD disparity led Ashley-Koch and colleagues to assess variation in both *MYH9* and *APOL1* in a well-characterized sickle cell disease cohort.[96] Because of the strong LD in the region of chromosome 22, which contains both *MYH9* and *APOL1*, they genotyped SNPs in both genes in order to determine if variants in either gene may be associated with risk of SCD nephropathy. Proteinuria and glomerular filtration rate (GFR) were the main clinical outcomes assessed for association with variation in *MYH9* and *APOL1*.

2.2.2 Methods and patient cohort characteristics

Data set. Five hundred and twenty one unrelated patients were enrolled as part of a multi-center study of genetic associations with clinical outcomes in SCD.

Participating institutions were Duke University, University of North Carolina at Chapel Hill, East Carolina University and Emory University Sickle Cell Centers. Patients enrolled were between ages 18–83 years. Diagnoses included Hb SS (n = 452), Hb $S\beta^0$ thalassemia (n = 18), Hb $S\beta^+$ thalassemia (n = 17), and Hb SC (n = 34). Mean and median ages were 33.9 and 32.0 years, respectively. Almost all patients (498, or 98.81%) identified themselves as non-Hispanic African-Americans, 2 (0.40%) as Hispanic Blacks, 1 (0.20%) as a non-Hispanic Caucasian, and 3 (0.60%) as Hispanic Caucasians. Two hundred and thirty subjects (44.15%) were male. Blood samples were obtained by routine phlebotomy at steady state (when patients were not experiencing a SCD crisis). All patients were also evaluated for proteinuria by a standard urine dipstick analysis. Individuals were categorized as having proteinuria if the dipstick analysis results were 1+ to 4+, and as not having proteinuria if the dipstick analysis demonstrated no protein or trace amounts. Because of the historical nature of the data set, it was not possible to obtain a more accurate measure of proteinuria, such as collection of 24-h urine samples. Despite the availability of more accurate methods of detecting proteinuria, the sensitivity and specificity of dipstick to detect microalbuminuria has been estimated to be 70% and 83%, respectively.[97] The prevalence of proteinuria in the data set was 26.9%, which is consistent with the 20–30% previously reported in SCD.[98] Genetic analysis used the occurrence of proteinuria as the dependent variable, as there was not complete information regarding SCD nephropathy in the data set and proteinuria is a key

component of the nephropathy disease process. GFR was calculated from serum creatinine, serum urea nitrogen, and serum albumin concentrations, adjusted for age, race, and gender using the 'Modification of Diet in Renal Disease' (MDRD) study definition described elsewhere.[99]

Genotyping. Twenty-six haplotype tagging SNPs in *MYH9* were selected using LDSelect v1.0,[100] based on data from the Yoruban population in the HapMap project (http:// hapmap.ncbi.nlm.nih.gov). In order to minimize redundancy among SNPs in high LD, a single SNP was selected to represent each haplotype block, as defined by $r^2 > 0.64$. SNPs were also required to have a minor allele frequency >0.05 so that there would be reasonable power to detect associations in the data set. Two SNPs (rs73889319 and rs71785313) in *APOL1* that had been identified as being most strongly associated with focal segmental glomerulosclerosis were also typed.

Although the focus of this study was a targeted candidate region analysis, whole genome data from the Illumina 610 chip existed on 480 of the subjects in this data set. Therefore, the ancestry of these individuals was analyzed with principal component analysis (PCA). To utilize SNPs with differing minor allele frequency in the African and European parent populations, SNPs were cross-referenced with the 610 chip and the Illumina African American Admixture panel. 392 overlapping SNPs were then used to estimate the percentage of European admixture for each individual with the linkage model in STRUCTURE.[101]

2.2.3 Results

Proteinuria. Analysis of the data set using the genotype test resulted in identification of 10 SNPs that were nominally associated with proteinuria ($P < 0.05$); eight of these SNPs met the corrected multiple comparison threshold ($P < 0.0025$) (Figure 3). One SNP was in the APOL1 gene (rs73885319, $P = 0.0004$) and the remaining seven SNPs were in the MYH9 gene (rs11912763, $P = 0.0001$; rs16996648, $P = 0.0021$; rs5750248, $P = 0.0021$; rs1557529, $P = 0.0006$; rs8141189, $P = 0.0004$; rs1005570, $P = 0.0015$; rs16996672, $P < 0.0001$; Table 3). The *APOL1* SNP that met the multiple comparison threshold (rs73885319), is a missense mutation (S342G) which is in strong LD with another missense mutation (I384M) that was not typed in this data set. These two SNPs have been previously referred to as the G1 tag that was associated with FSGS, as described above. The 6-bp deletion SNP in *APOL1* (rs71785313, termed G2) was also typed. This SNP was not associated as a main effect in the data set. However, because previous work had examined the *APOL1* G1 and G2 risk alleles jointly and assumed a different genetic model, the analyses of the *APOL1* gene was done using these previous assumptions. It was observed that those individuals with 2 risk alleles for G1 or G2 (G1/G1 or G1/G2 or G2/G2) were 3.4 times as likely to have proteinuria as those with none or one (either G1 or G2) risk allele ($P < 0.0001$). There was no difference in risk for proteinuria among those with no risk alleles and 1 risk allele. These findings are consistent with the recessive model reported in Genovese *et al.*, 2010. Of note, among the

individuals with 2 *APOL1* risk alleles, over half (52. 9%) were homozygous for the G1 risk allele, consistent with the observation that rs73995319 provided evidence for a main effect.

Among the *MYH9* SNPs that met the multiple comparison threshold, rs5750248 and rs11912763 were identified previously as representing the S-1 and F-1 risk haplotypes respectively, that were very strongly associated with kidney disease among African Americans and Hispanics.[91, 102] Despite an attempt to reduce LD in the *MYH9* gene by selecting haplotype tagging SNPs from the Yoruba population, there was some evidence of LD in the data set. One of the most significant SNPs, rs11912763, was in high LD ($r^2 = 0.72$) with rs16996648 and in moderate LD ($r^2 = 0.57$) with rs5756152. These correlations are reflected in the correlation among the association results, whereby both rs11912763 and rs16996648 were significantly associated with proteinuria, and rs5756152 approached significance.

Among the eight SNPs in *APOL1* and *MYH9* that met the multiple testing correction for significant association with the occurrence of proteinuria, the frequency of proteinuria among the individuals who were homozygous for the risk genotype at a particular SNP ranged from 35% to 50%, as compared to the frequency of proteinuria among the individuals who did not have that genotype, which ranged from 20% to 30%. Odds ratios for specific genotype comparisons are shown in Table 3.

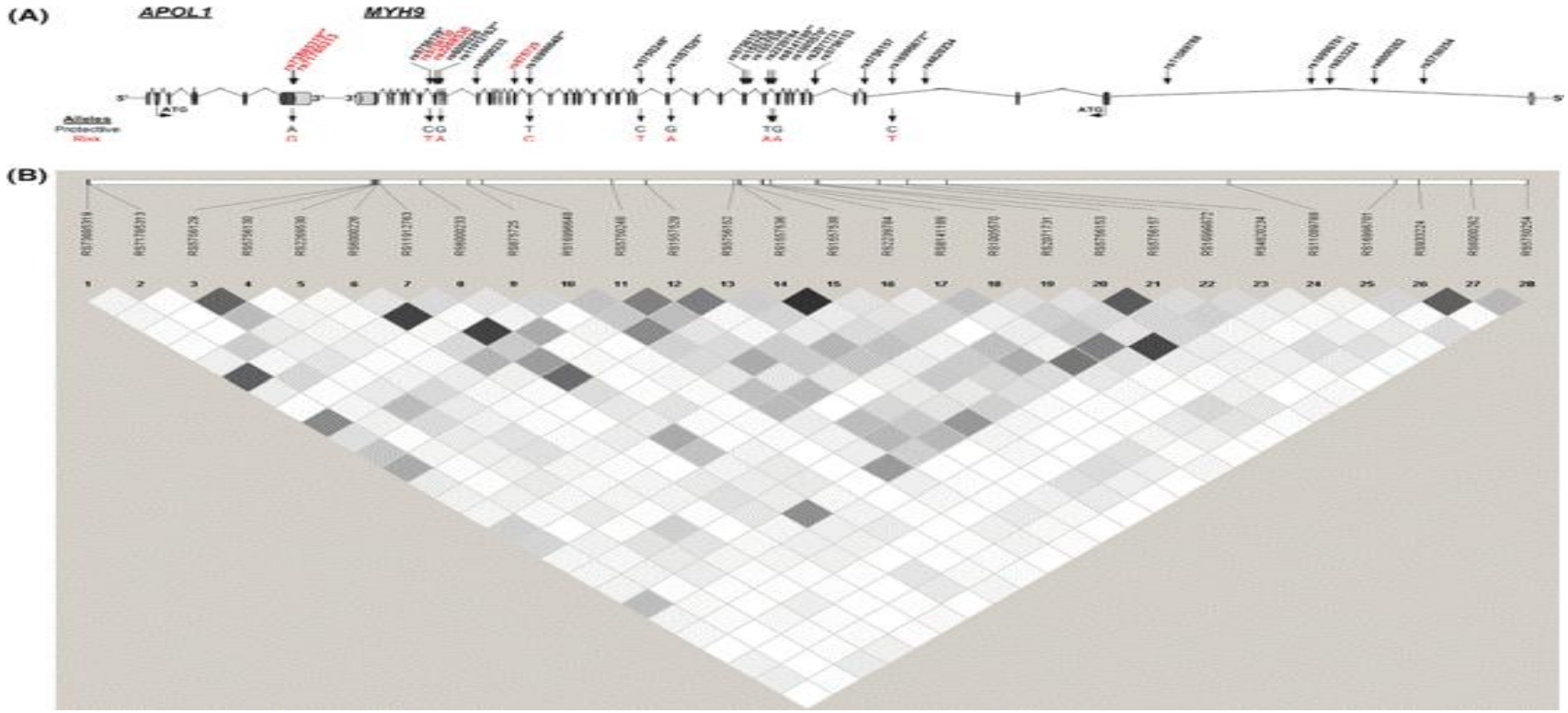

Figure 3: *MYH9* and *APOL1* genotyped SNPs and LD structure of the region in SCD patients.

(A) Structure of the *APOL1* and *MYH9* genes. Genotyped SNPs are shown in different colors: black – non-coding; red – coding. SNPs defining the *MYH9* risk haplotype, along with their risk alleles, are indicated below the gene structure diagram. *Nominally significant at $P < 0.05$; **Significant at threshold adjusted for multiple testing of $P < 0.0025$. (B) LD map for the 28 SNPs typed shown. Darker squares represent stronger LD. Figure is reprinted with permission, ©Ashley-Koch, A.E., *et al.* 2011. Originally published in *British J Haematol*. 155(3):386-94.

In order to reduce the dimensionality of the SNPs in the *MYH9* gene, haplotype association analysis was performed, using the 9 SNPs in the *MYH9* gene that were nominally significant. This analysis revealed that the risk haplotype (including the risk allele for all 9 SNPs) was the second most prevalent haplotype for *MYH9* in the study population. About 15% of the subjects in the data set had this haplotype, which was also significantly associated with proteinuria ($P = 0.0003$), such that those with the risk haplotype were about 2. 9 times as likely to have proteinuria as those with the most frequent haplotype, which had a prevalence of 20%. Those with the risk haplotype were also 1. 8 times more likely to have proteinuria as those with rarer haplotypes, which were grouped together for the purpose of analysis. Analysis by haplotype was also adjusted for age, because age was significantly associated with proteinuria ($P = 0.00001$).

Given reports that SCD patients with a more severe form of the condition (Hb SS and Hb $S\beta^0$) have a higher prevalence of proteinuria than other SCD forms, a second analysis was performed on only those individuals and tests for association were repeated. Using the genotype test, the results were similar. Eight SNPs were nominally significant ($P < 0.05$), with 4 reaching the corrected multiple comparison threshold [$P < 0.0025$; rs73885319 (*APOL1*), rs11912763 (*MYH9*), rs8141189 (*MYH9*) and rs16996672 (*MYH9*)]. Hb SS and Hb $S\beta^0$ individuals made up the majority (90.13%) of the data set, which probably explains the similarity to the results in the overall data set.

Glomerular filtration rate. Because of the known relationship between proteinuria and GFR, this relationship was also examined in the data set and it was found that GFR was inversely correlated with proteinuria ($r = -0.25$, $P < 0.0001$), such that for every one-unit decrease in GFR, the odds of having proteinuria increased (OR = 1.03, CI 1.02–1.04). Furthermore, they examined whether or not the *APOL1* and *MYH9* SNPs were associated with GFR, but did not detect any associations in unadjusted analyses. However, when we controlled for age, nominal associations were observed with rs73885319 (*APOL1*, $P = 0.02$), rs11912763 (*MYH9*, $P = 0.03$), and rs933224 (*MYH9*, $P = 0.002$). When haplotype analysis was performed for the *MYH9* gene and controlled for age, a significant association with GFR was not detected, although there was a trend ($P = 0.11$). Similarly, when examining the G1/G2 *APOL1* recessive model, there was a trend whereby individuals with two risk alleles had lower GFR than individuals with 0 or 1 risk allele, but this was not statistically significant ($P = 0.12$).

Complex genetic models. Given that the previous work in non-SCD populations were providing stronger evidence for association between ESRD and *APOL1* rather than *MYH9*,[92, 103] multiple regression analysis was conducted. The analysis was repeated, where proteinuria was predicted as a function of each of the nine nominally significant *MYH9* SNPs (Table 3), while controlling both for age and the G1/G2 *APOL1* recessive model described above. Unlike in the previous reports, four of the *MYH9* SNPs remained significant predictors of proteinuria, even when *APOL1* was a covariate.

Table 3: Significant *APOL1* and *MYH9* SNPs associated with proteinuria in a SCD cohort.

Gene	SNP	Genotype	Frequency of proteinuria	Odds Ratio (CI)	P-value for comparison	Global nominal P-value
APOL1	rs73885319	AA	0.214	0.205 (0.092, 0.457)	0.0001	0.0004
		AG	0.315	0.306 (0.132, 0.707)	0.0056	
		GG	0.500	0.670 (0.421, 1.067)	0.0917	
MYH9	rs11912763	AA	0.539	5.675 (2.423, 13.29)	<0.0001	0.0001
		AG	0.319	1.692 (1.063, 2.694)	0.027	
		GG	0.216	3.353 (1.392, 8.081)	0.007	
MYH9	rs16996648	CC	0.450	3.338 (1.632, 6.827)	0.001	0.0021
		CT	0.308	1.230 (1.031, 2.556)	0.037	
		TT	0.213	2.057 (0.992, 4.262)	0.053	
MYH9	rs5750248	CC	0.159	0.320 (0.162, 0.633)	0.001	0.0021
		CT	0.247	0.560 (0.352, 0.893)	0.015	
		TT	0.356	0.572 (0.295, 1.108)	0.098	
MYH9	rs8141189	AA	0.475	3.995 (1.936, 8.244)	0.0002	0.0004
		AT	0.310	1.752 (1.113, 2.759)	0.0154	
		TT	0.200	2.280 (1.105, 4.705)	0.0258	
MYH9	rs1005570	AA	0.370	2.996 (1.626, 5.519)	0.0004	0.0015
		AG	0.294	2.013 (1.200, 3.378)	0.0081	
		GG	0.173	1.488 (0.875, 2.531)	0.1421	
MYH9	rs16996672	CC	0.192	0.191 (0.091, 0.401)	<0.0001	0.00003
		CT	0.308	0.361 (0.173, 0.753)	0.0066	
		TT	0.526	0.527 (0.334, 0.833)	0.0061	

Table is adapted with permission, ©Ashley-Koch, A.E., *et al.* 2011. Originally published in *British J Haematol*. 155(3):386-94.

In these multiple regression analyses, the most significant association in the *MYH9* gene remained with rs16996672 (P = 0.001). Furthermore, in these analyses, the evidence for association with *APOL1* remained significant. This analysis was also repeated using the *MYH9* risk haplotype and the *APOL1* recessive model. In this age-adjusted analysis, both the *MYH9* risk haplotype (P = 0.008) and *APOL1* (P = 0.02) were significant predictors of the occurrence of proteinuria. Interestingly, when GFR was modelled as a function of the *MYH9* risk haplotype and the *APOL1* recessive model, there was evidence for a highly significant interaction between the two risk factors ($P = 3 \times 10^{-14}$). Specifically, among individuals with 0 or 1 *APOL1* risk alleles, the frequency of the *MYH9* risk haplotype was 12% but among the individuals with 2 *APOL1* risk alleles, the frequency of the *MYH9* risk haplotype was 43%. Thus, in the SCD population, there is evidence for association with both the *MYH9* and *APOL1* genes predicting kidney dysfunction.

2.2.4 Summary of *MYH9* and *APOL1* associations in sickle cell disease nephropathy

The work by Ashley-Koch *et al*. was the first identification of variation at the chromosome 22q12 region with clinical risk factors for SCDN, providing more evidence for association at this region with multiple forms of CKD. Thus, identification of SNPs in *MYH9* and *APOL1* that are significantly associated with proteinuria was quite intriguing. Although the association with ESRD had originally been detected with the *MYH9* gene, subsequent work has suggested that this association is probably due to a

genetic 'hitchhiking' effect with the *APOL1* gene, due to the strong LD between these two genes and the selective pressures believed to have perpetuated certain genetic variants of *APOL1*. Unlike in previous studies in non-SCD African American CKD, this analysis in a SCD population provides evidence that *both* genes (*MYH9* and *APOL1*) are independent risk factors for proteinuria. The discrepancies between these results and previous studies suggested that SCDN etiology may be unique compared to other forms of CKD in African American populations and that genetic factors associated with risk for SCDN may differ as well.

The second important finding by Ashley-Koch *et al.* was found in the interaction of *MYH9* and *APOL1* for SCDN risk. In this analysis, statistical models predicting proteinuria provided evidence for main effects of both genes, when both were included in the model. This suggests that for proteinuria, each gene is an independent risk factor. In contrast, statistical models predicting GFR did not provide strong evidence for independent main effects of the genes, but provided very strong evidence of an interaction between the two genes. However, in the absence of sophisticated functional work, the specific role of each gene remained unclear.

2.3 Initial functional characterization of MYH9 and APOL1 and the evolution of the APOL gene cluster

2.3.1 Studies into *MYH9* and *MYH9*-Related Disorders

In mammalian systems, there exists three different non-muscle myosin heavy chain (NMHC) molecules, each encoded by its respective gene: *MYH9, MYH10,* and

MYH14 (NMHC-IIA, NMHC-IIB, and NMHC-IIC, respectively).[104, 105] All non-muscle myosin II proteins are critically important for many aspects of cell integrity, including: contractility, morphology, cytokinesis polarity, and cell migration.[106-110] Most cells throughout the body express multiple NMHC-II isoforms, except for platelets and leukocytes, which exclusively express NMHC-IIA.[111, 112] This is part may be why nearly 40 *MYH9* coding mutations have been linked to several overlapping syndromes, all of which display macrothrombocytopenia.[113-115] As discussed above, these syndromes (May-Hegglin, Sebastian, Fechtner, and Eptsein) have been classified as '*MYH9*-Related Disorders', and display other variable pathologies including cataracts, deafness, or nephritis (Table 4). It was later found that *MYH9* is expressed in podocytes, nephric tubular cells, mesangial cells and endothelial cells, suggesting a relevant role to the kidney pathology sometimes seen in these patients.[116] Overall, kidney abnormalities are seen in about 30%-70% of *MYH9*-Related Disorders, and patients may present hematuria (with or without proteinuria) segmental glomerulosclerosis, glomerular basement membrane thickening, and tubulointerstitial disease.[117, 118]

Table 4: Frequent pathologies displayed in *MYH9*-Related Disorders

	May-Hegglin	Sebastian	Fechtner	Epstein
Large platelets	Yes	Yes	Yes	Yes
Thrombocytopenia	Yes	Yes	Yes	Yes
Leukocyte inclusion	Yes	Yes	Yes	--
Deafness	--	--	Yes	Yes
Cataracts	--	--	Yes	--
Nephritis	--	--	Yes	Yes

Several studies have attempted to address the functional effect of *MYH9* mutations on glomerular pathology. However, one of the main challenges has been the identification of an appropriate animal model. First studied in mice, it was found that *Myh9* knockouts die at an early embryonic age, resulting from a failure of germ layer organization.[119] This finding also prohibited the use of tissue-specific knockouts as the cells would simply fail to develop. Furthermore, it was found that heterozygous *Myh9* mouse knockouts are viable without any observable kidney abnormalities.[120] More recent studies in mice have shown a more promising link. Knock-in experiments, in which mice express the most common mutation present in *MYH9*-Related Disorders (R702C +/-), display albuminuria as well as glomerulosclerosis when aged.[121] In addition, homozygous point-mutation knock-in mouse lines containing two other recurring mutations in patients (D1424N and E1841K) show various kidney abnormalities including focal segmental glomerulosclerosis and progressive albuminuria.[122] Finally, and probably the most contributing study, Müller and colleagues use the zebrafish as a model to assess the role and function of *myh9* in the glomerulus.[123] In this study they showed specific expression of *myh9* in podocytes, mesangial cells and endothelial cells.[123] Upon morpholino-induced knockdown of *myh9*, they found extensive malformations in the glomerular capillary tuft, podocyte effacement, glomerular basement membrane thickening, and compromised glomerular barrier function resulting in decreased GFR.[123] Interestingly, they did not observe any defects to platelet function in *myh9* morphants.[123]

In summary, these *in vivo* studies have given us great insight into the possible role of *MYH9* in glomerular involvement, suggesting that it has an essential role in the development of the pronephric glomerulus in zebrafish as well as the maintenance of the glomerular barrier in aged mice. However, despite these studies, the molecular mechanism by which *MYH9* may induce kidney pathology remains unknown. In addition, the observation that family members with *MYH9*-Related Disorders have the same recurring *MYH9* mutation, but differ in renal presentation, suggests that mutations in *MYH9* may not be the only determinant for the development of the kidney phenotype. This may indicate that other genes may be involved and/or *MYH9* may be interacting with other partners to induce glomerulopathy. Finally, in respect to recent associations of the 22q12 locus (*MYH9*/*APOL1*) with African American CKD, these *in vivo* studies suggest that although the strongest association may reside in *APOL1* G1/G2, the apparent biological function of *MYH9* should not be ignored and may play a part in CKD progression.

2.3.2 The apolipoprotein L gene cluster.

The apolipoprotein L family consist of six genes grouped together in a 620 kb region on chromosome 22 (22q12.3), and includes *APOL1, APOL2, APOL3, APOL4, APOL5* and *APOL6.* The entire cluster resides just upstream of *MYH9*, with *APOL1-4* only about 100 kb away and *APOL5* and *6* about 400 kb farther upstream (Figure 4). *APOL2*, *3* and *4* are transcribed on the reverse strand and *APOL1*, *5* and *6* are transcribed

on the forward strand. Several studies have attempted to address the evolutionary history of this gene cluster in order to give further insight into the apparent function of each of these proteins.[124-126] By assessing phylogenetic relationships in primates, it has been shown that the entire family is highly polymorphic with several gain/loss events between species.[126] Interestingly, all *APOL* genes share a large exon that contains about

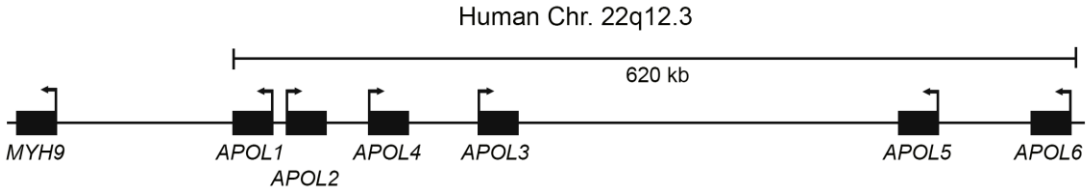

Figure 4: Genome organization of *MYH9* and the *APOL* gene cluster.

All apolipoprotein L genes reside within a 620 kb region on human chromosome 22q12.3. The cluster has evolved through several gain/loss events in primates and recent human evolution.

70%-80% of the total coding sequence, with the remaining exons (from one to four, depending on the gene) residing at the N-terminus and differ between genes and isoforms.[126] Finally, using Phylogenetic Analysis by Maximum Likelihood (PAML), Smith and Malik found evidence for positive selection in all primate *APOL* proteins with the exception of *APOL5*. The highest PAML p-value was actually found in *APOL6* among primates and even showed evidence of positive selection in recent human evolution.[126] These studies provided initial evidence that these genes may be clinically important as they showed a history of positive selection within the *APOL* gene cluster, possibly due to human disease resistance or susceptibility.

2.3.3 Initial studies into the role of *APOL1*

By far the most studied apolipoprotein is *APOL1*, which is known to confer resistance to *trypanosoma brucei* by encoding a trypanolytic factor in humans and some primate species.[94, 126] In fact, the method by which *APOL1* lyses trypanosomes gives insight into its protein domains and overall protein function. It has been shown to associate with high-density lipoprotein (HDL) particles in human blood, as it is unique compared to the other *APOL* proteins in that it is the only member to contain an N-terminal signal peptide and so is secreted outside the cell.[124, 127] *T. brucei*, after it enters the host, endocytose HDL particles to obtain various lipids and iron from the bloodstream.[128] As the parasite traffics these HDL particles to the lysosome it induces a pH change, which causes a conformational change in the membrane addressing domain (MAD) of *APOL1.* This then causes *APOL1* to bind to the lysosomal membrane and create anion-specific pores by way of its pore-forming protein domain, which lead to lysosomal swelling and eventually trypanolysis.[93]

APOL1 is also unique among the other *APOL* proteins in that *APOL1* contains a BH3 domain, which is characteristic of the BH3-family of pro-apoptotic genes, and so allows it to initiate programmed cell-death in host cells.[129] However, it is important to note that BH3-proteins only function in response to particular stimuli, and often require a set of factors to bind or unbind in order to induce cell-death.[130] Interestingly, and in-line with its role in cell death, Wan and colleagues reported that intracellular

overexpression of *APOL1* causes autophagic death of human cells.[131] This finding is actually consistent with its role in trypanolysis, in that both mechanisms use endosome trafficking to the lysosome.[132]

Due to its binding of HDL, a role in lipid metabolism has also been proposed for *APOL1*. A sterol-responsive element in its promoter region and amphipathic helices in its secondary structure seem to provide further evidence for this assumption.[127] In addition, one report found that individuals with two *APOL1* risk variants had a reduction in HDL particles compared to individuals with one or no risk variants.[133] However, total HDL concentration was not different between these same two groups, suggesting that *APOL1* may only alter specific fractions of HDL.

Finally, localization and expression studies yield some insight into the possible role of APOL1 protein. It has been localized to the vasculature endothelium in normal human kidney sections, as well as podocytes and the proximal tubule.[125, 134] However, it has not yet been determined if *APOL1* is transcribed in these cell types or taken up in the circulation. Some transplantation studies do suggest that African American donors with two *APOL1* risk variants had a shorter graft survival compared to kidneys obtained from individuals with one or zero risk variants.[135] This finding provides tentative evidence that the risk for progression of kidney disease may be passed on by *APOL1* expressed in the kidney. However, the mechanisms by which *APOL1* and its risk variants lead to kidney disease and CKD progression remain unclear.

3. *In Vivo* Functional Studies into APOL1 Associated Nephropathies

This chapter is adapted from and includes figures and tables from the published manuscript:

Anderson, B.R., Howell, D.N., Soldano, K., Garrett, M.E., Katsanis, N., Telen, M.J., Davis, E.E., and Ashley-Koch, A.E. *In Vivo* Modeling Implicates APOL1 in Nephropathy: Evidence for Dominant Negative Effects and Epistasis under Anemic Stress. PLoS Genetics 11(7): e1005349. doi:10.1371/journal.pgen.1005349 (2015)

3.1 Introduction

Despite the genetic findings and the association of the 22q12 locus with increased risk of multiple forms of CKD in African American populations, there is a dearth of functional data to inform directly whether *MYH9* or *APOL1* is the driver of this genetic association. In mice, homozygous *Myh9* knockouts die at an early embryonic stage[119], and heterozygotes appear viable without any detected abnormalities.[120] However, subsequent studies have demonstrated that knock-in mutants display renal glomerulosclerosis, while podocyte-specific deletion of *Myh9* predisposes mice to glomerulopathy.[121, 122, 136] In zebrafish, *myh9* is required for the normal development of the glomerulus; morpholino (MO)-induced *myh9* suppression results in non-uniform podocyte foot processes and glomerular basement membrane thickening.[123] In contrast, the possible relevance of *APOL1* to CKD is derived primarily from *in vitro* work: cellular localization studies of *APOL1* in nondiabetic kidney disease patient biopsies suggest an implication in arteriopathy[134, 137], while overexpression of *APOL1* and its risk alleles enhance podocyte necrosis *in vitro*.[138] Similar toxicity has also been observed in *Xenopus laevis* oocytes.[139]

Here, we used zebrafish as an *in vivo* model to study the consequences of gene perturbation and potential synergistic effects of *APOL1* and *MYH9* in kidney disease. Although the zebrafish pronephros is a simplified kidney, the structure and function of the larval glomerulus is similar to that of humans and represents a tractable model in

which to study *apol1* and *myh9*.[140, 141] In this report, we provide insight into the role of *apol1* in glomerular development and pronephric filtration in zebrafish embryos, as well as the effects of *APOL1* G1 and G2 allelic expression. Moreover, we provide functional evidence for an interaction between *myh9* and *apol1* under anemic stress conditions. Overall, these data implicate both *MYH9* and *APOL1* as significant biological contributors to nondiabetic nephropathy and intimate context-dependent roles in disease pathology.

3.2 Results

3.2.1 Knockdown of zebrafish apol1 results in pericardial edema, compromised glomerular filtration, and disruption of the glomerular ultrastructure.

The apolipoprotein L family of genes evolved rapidly in humans and some non-human primates.[125, 142] However, using BLAST and reciprocal BLAST searches against the *D. rerio* and *H. sapiens* genomes, we identified a single *D. rerio* locus encoding a protein of unknown function (chr2:37,674,122-37,676,731 Zv9; NCBI Ref: NP_001025309.1 ; 38% identity, 46% similarity on the amino acid level) as a possible unique functional ancestral ortholog to the human apolipoprotein L family (Figure 5, A-D). To explore the function of this transcript in developing zebrafish, we first asked whether the candidate *apol1* ortholog is expressed in a temporal manner amenable to transient assays of renal development and function. RT-PCR analysis of cDNA

generated from wild-type (WT) whole-larval total RNA collected at three days post-fertilization (dpf) and 5 dpf showed

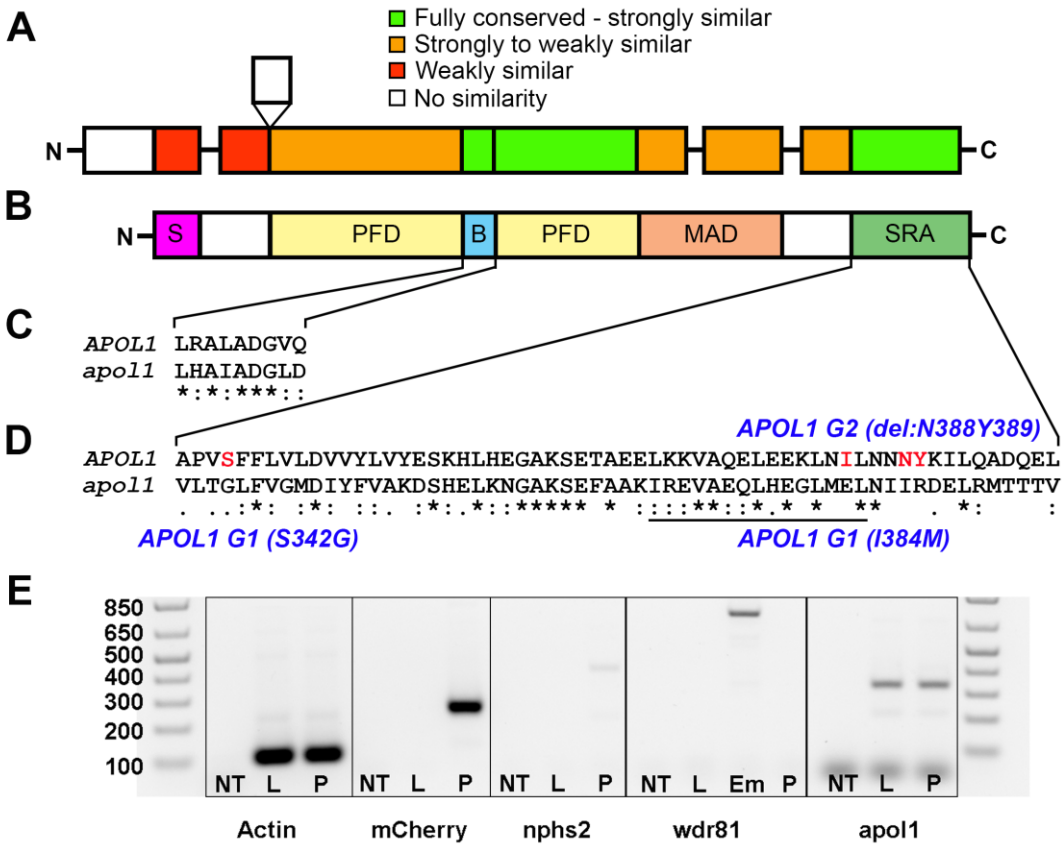

Figure 5: Comparison of APOL1 human and zebrafish protein sequences and relevance to the zebrafish kidney.

Protein domain schematic of (A) zebrafish APOL1 and (B) human APOL1 is shown, with zebrafish domains aligned to the human protein and coded based on summarized consensus scores (Gonnet PAM 250 matrix, Clustal Omega, Cambridge, UK; S, secretory domain, PFD, pore-forming domain, B, BH3 domain, MAD, membrane-addressing domain, SRA, serum resistance-associated binding domain). Prominent regions of the human and zebrafish alignments are expanded, including the (C) BH3 domain and (D) SRA binding domain, and consensus symbols are displayed (* (asterisk), fully conserved; : (colon), >0.5 in the Gonnet PAM 250 matrix; . (period), =<0.5 in the Gonnet PAM 250 matrix). The leucine zipper domain (codons 365-392 in APOL1, underline), and the location of the G1 and G2 risk alleles in CKD in African Americans (S342G/I384M and ΔN388Y389) are highlighted in red. (E) Podocytes from adult glomeruli of pod::NTR-mCherry zebrafish were flow-sorted. *apol1* is expressed in fluorescence-activated cell sorted (FACS) podocytes and the adult liver. FACS podocytes also express zebrafish podocin

(*nphs2*) but a purkinje-cell marker, *wdr81*[143], was undetectable. NT = non-template reverse transcription control; L = dissected adult liver cells from pod::NTR-mCherry zebrafish; P = fluorescence-activated cell sorted podocytes from dissected glomeruli of pod::NTR-mCherry zebrafish; Em = 5 dpf whole zebrafish embryo cDNA.

expression at time points corresponding to the formation of the pronephros (data not shown). Additionally, we detected *apol1* expression in flow-sorted podocyte fractions harvested from glomeruli of *pod*::NTR-mCherry adult zebrafish (Figure 5, E).[144]

To test the effects of *apol1* suppression, we designed a translation-blocking morpholino (MO) targeting the candidate zebrafish *apol1* locus (*apol1*-MO) and we injected increasing doses into embryos at the one to four cell stage (*n*=49-65 embryos/injection; repeated three times). Masked scoring for morphological defects at 5 dpf revealed a dose-dependent increase of the percent of larvae displaying pericardial and yolk sac edema, a phenotype that has been implicated previously in glomerular filtration defects (Figure 6, A-C).[140, 145] Co-injection of WT *APOL1* human mRNA (GenBank Accession: BC112943.1 ; 100 pg/nl) rescued significantly the edema caused by *apol1* suppression ($p<0.0001$; Figure 6, D), arguing not only that the phenotype was unlikely to be a non-specific toxic effect of the MO, but also that the zebrafish locus we targeted is the ortholog of the human transcript. Additionally, we observed a significant decrease in endogenous *APOL1* protein expression in *apol1*-MO injected zebrafish embryos ($p = 0.026$), which is restored to normal levels upon co-injection with wild-type human *APOL1* mRNA (Figure 7, A-B). Finally, co-injection of human mRNA encoding

other human apolipoprotein L members (*APOL2*, *APOL3*, *APOL4*, *APOL5*, and *APOL6*) with *apol1* MO did not rescue the edema formation of *apol1* morphants (Figure 8, A-F).

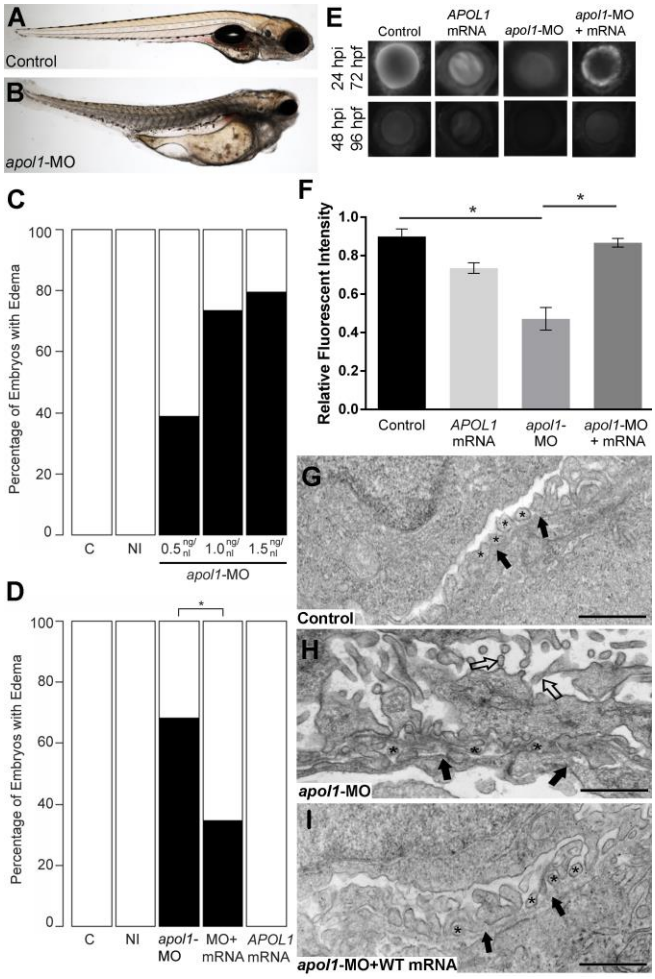

Figure 6: *apol1* morphant zebrafish embryos display generalized edema and glomerular filtration defects indicative of nephropathy.

Representative live images of (A) sham-injected control larvae, and (B) *apol1* morpholino (MO) injected larvae at 5 dpf. *apol1* morphants display pericardial and yolk sac edema. (C) Injection of increasing doses of *apol1*-MO demonstrate dose-dependent effects when scored for generalized edema (n=35-65 embryos/injection; repeated three times) compared to control larvae at 5 dpf. *apol1* morpholino injected embryos were complemented with the respective human mRNA to *APOL1* (100pg/nl) and scored for generalized edema at 5 dpf. (D) Ectopic expression of *APOL1*

significantly rescues the edema phenotype observed in *apol1* morphants (1.0ng/nl dose). We observed no significant phenotypes when *APOL1* human mRNA is injected alone. 70kDa dextran-FITC conjugate was injected into the cardiac venous sinus of 48 hpf zebrafish larvae and fluorescence intensity in the eye vasculature was measured at 2, 12 and 36 hpi. (E) Representative eye image series of zebrafish larvae for each injection group show a relatively stable or a decrease in fluorescence intensity over time compared to sham-injected controls. (F) Line graphs summarize the fluorescence changes observed for each injection group for apol1 morphant larvae. Reduction in fluorescence intensity over the pupil was calculated relative to the 2 hpi. time point. (E, F) apol1 morphants display increased glomerular clearance of 70kDa dextran-FITC compared to control embryos over time, indicative of compromised glomerular filtration. These defects were rescued significantly when MO was co-injected with orthologous human mRNA. (G-I) compared to (G) sham-injected controls, the glomerular ultrastructure of (H) *apol1* morphant zebrafish are devoid of podocyte foot process (* asterisks), although the glomerular basement membrane (arrowheads) appears normal. (I) Ultrastructure defects are rescued upon co-injection of human wild-type mRNA (100pg). Scale bar, 500nm. White bars, normal; black bars, edema. MO concentrations are in μg/μl, with 1nl injected into each embryo. C, sham-injected control; NI, non-injected control. Dextran values are in relative fluorescent intensity, mean ± SE. Control, sham-injected control (n=29); MO, *apol1* morpholino injected (n=26); apol1-MO+mRNA (n=28). h.p.f., hours post-fertilization; h.p.i., hours post-injection. *p<0.001.

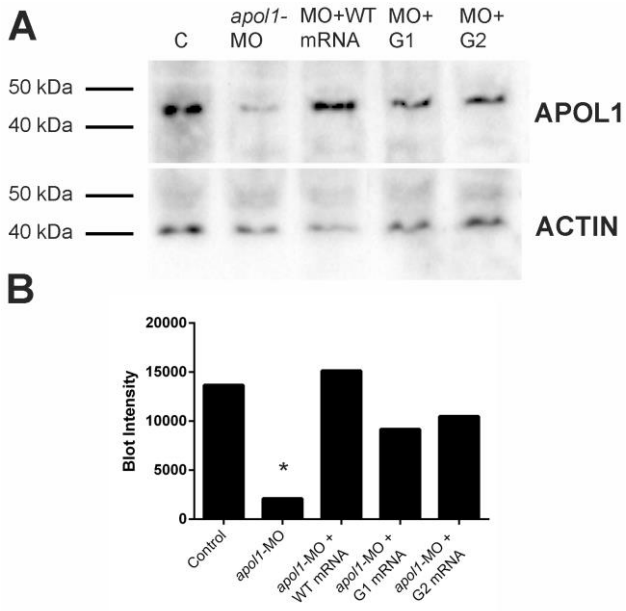

Figure 7: Characterization of APOL1 protein levels in *apol1*-MO and *APOL1* RNA-injected embryos.

Protein lysates from zebrafish embryos injected with *apol1*-MO (1.0ng/nl) alone or co-injected with either wild-type, G1, or G2 *APOL1* human mRNA (100pg) were isolated from 2 dpf embryos. (A) APOL1 protein levels were assessed by Western blot (Abcam EPR2907) and (B) pixel intensity normalized to ACTIN was calculated for comparison. (A-B) Embryos injected with

translation-blocking *apol1*-MO display a significant reduction in APOL1 protein expression compared to non-injected controls, suggesting cross-reactivity with zebrafish APOL1 and efficiency of the apol1 MO to block translation. Protein levels are restored to control levels upon co-injection of wild-type, G1, or G2 *APOL1* human mRNA. Blot shown is a representation of four independent experiments. Lane 1, non-injected control; Lane 2, *apol1*- MO injected; Lane 3, *apol1*-MO + wild-type *APOL1* human mRNA; Lane 4, *apol1*-MO + G1 *APOL1* human mRNA; Lane 5, *apol1*-MO + G2 *APOL1* human mRNA. *p = 0.026.

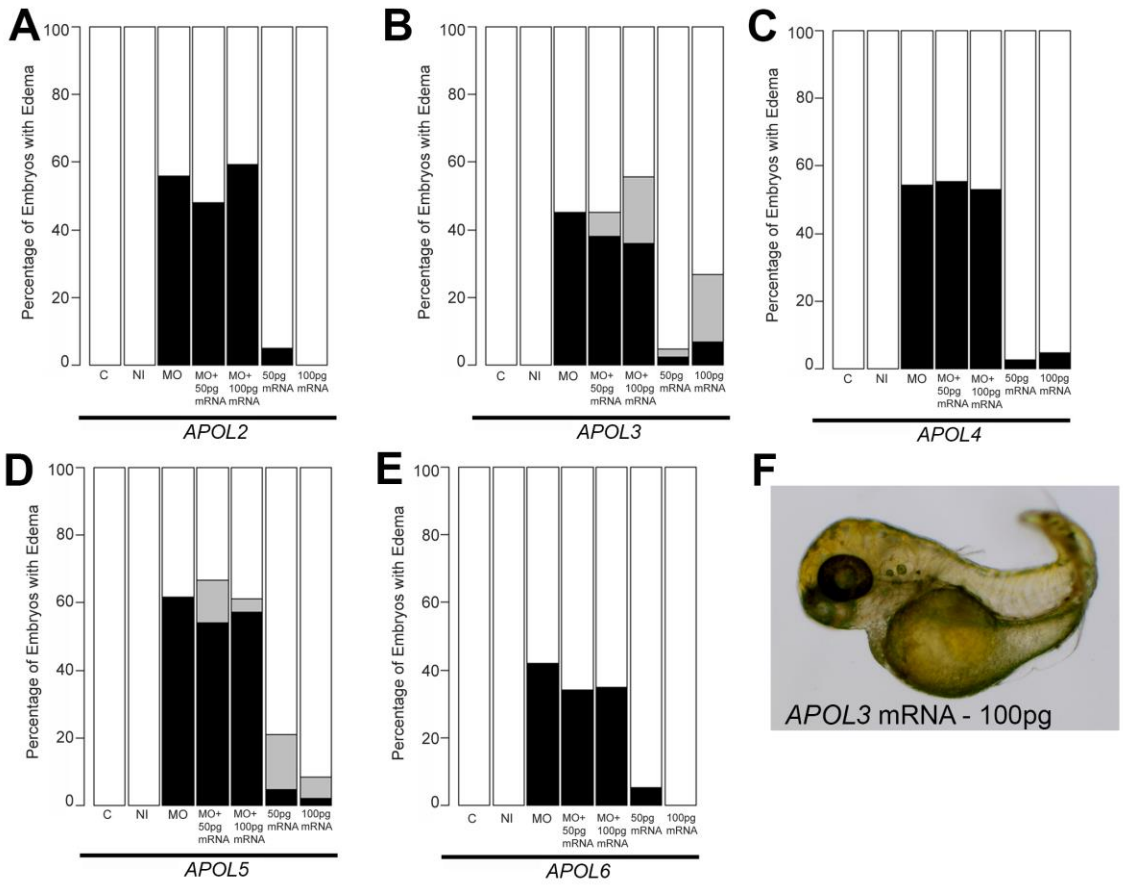

Figure 8: Complementation of zebrafish *apol1* morphants with other members of the human *APOL* gene cluster.

(A-E) Human mRNA corresponding to *APOL2, APOL3, APOL4, APOL5,* and *APOL6* (100pg/nl) were each co-injected with *apol1* MO and scored for edema at 5 dpf. Ectopic expression of none of the other members of the human *APOL* gene cluster were able to rescue significantly edema formation in developing embryos. (F) We observed a novel body axis phenotype in embryos injected with either *APOL3* or *APOL5* alone, although this did not seem to be relevant to kidney dysfunction. White bars, normal; black bars, edema; grey bars,

adverse. C, sham-injected control; NI, non-injected control; n=32-68 embryos/injection batch; masked scoring.

Furthermore, as an additional test of the specificity of *apol1* perturbation to edema formation, we induced microdeletions in exon 3 of *apol1* using the CRISPR/Cas9 system (Figure 9, A-C).[146, 147] Injection of guide RNA and CAS9 protein into one-cell stage embryos reproduced the edema phenotype (scored in founders, F0) seen in *apol1* morphants (n = 26–38 embryos/injection, repeated three times; $p<0.001$; Figure 9, D)

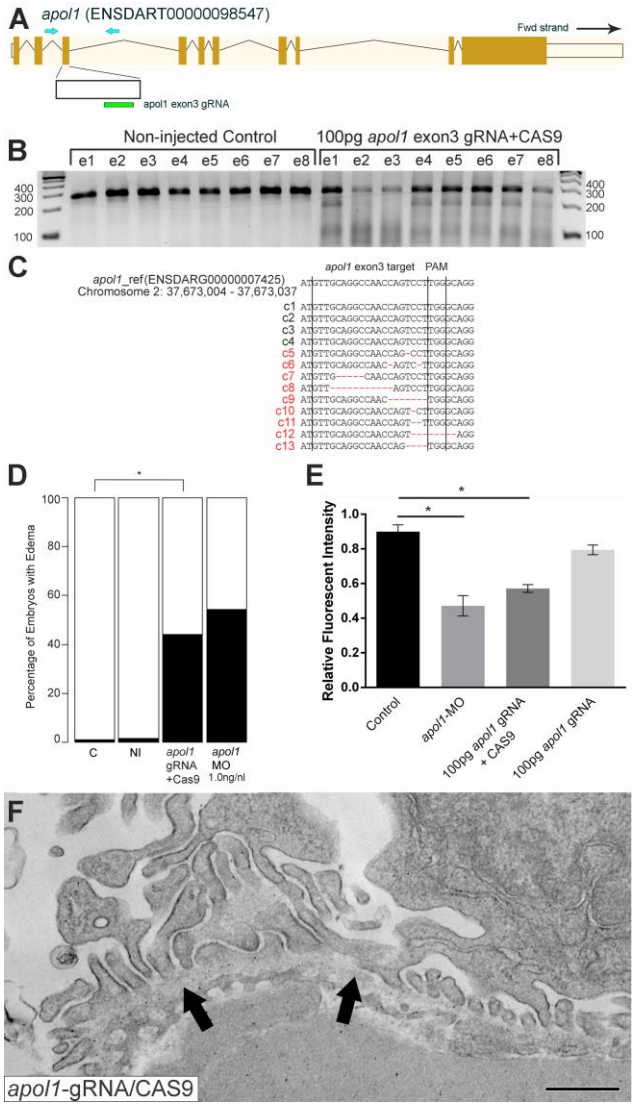

Figure 9: apol1-CRISPR F0 zebrafish embryos reproduce phenotypes observed in apol1 morphants.

Schematic of the zebrafish apol1 locus and location of the guide RNA (gRNA) target used for *apol1*-CRISPR experiments; the primers used to PCR-amplify the target region are shown (arrowheads). (B) At 1 dpf, a representative sampling of 8 founders and 8 non-injected controls were selected and subjected to T7 endonuclease 1 (T7E1) assay. The appearance of T7E1 fragments at ~180bp indicate positive gRNA targeting of exon 3 in the *apol1* locus. No T7E1 fragments were detected in non-injected control embryos. In total, 25 out of 41 founders subjected to T7E1 assay showed the presence of T7E1 fragments, indicating that ~61% of founders have insertion/deletions (indels) in the exon 3 region of *apol1*. (C) Multiple sequence alignment of *apol1* reference sequence (ENSDARG00000007425) to apol1-CRISPR variants generated from PCR

amplification and subsequent TA cloning and sequencing of two representative apol1-gRNA/CAS9 injected founders. 13 PCR-cloned sequences are shown, representing four wild-type variants (c1-4) and all indel types detected among 50 PCR-clones (c5-13). Of 50 total PCR-clones sequenced, 31 showed detectable indels, representing an estimated 62% mosaicism in *apol1*-CRISPR/CAS9 injected founders. Lines mark the specific sequence targeted by the apol1-gRNA (exon3) and the location of the PAM recognition motif (i.e. TGG). (D) *apol1*-gRNA and CAS9 co-injected embryos were scored for edema formation at 5 dpf (n = 26–31 embryos/injection, repeated three times; *p <0.001). (E) *apol1*-gRNA and CAS9 co-injected embryos display increased glomerular clearance of 70kDa dextran-FITC compared to control embryos over time, similar to that of apol1-MO injected embryos (*p <0.001). Bar graphs summarize the changes for each injection group. Dextran values are in relative fluorescence intensity, mean ± SE. Control, sham-injected control (n = 19–21); *apol1*-gRNA+CAS9 (n = 11 –17); *apol1*-gRNA alone (n = 13–14), repeated 2 times. (F) *apol1*-CRISPR/CAS9 injected embryos display podocyte foot process effacement at 5 dpf, similar to that of apol1 morphant larvae. Ultrastructural defects appear less severe when compared to apol1-MO injected embryos, however, including less foot process effacement and the absence of microvilli in the urinary space. Filled arrowheads, glomerular basement membrane. Scale bar, 500nm.

To test whether the generalized edema phenotype was relevant to nephropathy, we assessed the integrity of the glomerular filtration barrier in *apol1* morphants and F0 mutants as described.[145] First, we injected 70-kDa FITC-labeled dextran into the cardiac venous sinus of larvae at 48 hours post-fertilization (hpf). After injection, the eye vasculature was imaged at 24 and 48 hours post-injection (hpi; Figure 6, E-F). We quantified the average fluorescence intensity (ImageJ) and calculated changes in intensity at 48 hpi relative to the 24 hpi measurements. *apol1* morphant larvae display a significant reduction in circulating 70-kDa dextran compared to controls (n = 26; p = 4.44x10-4; MO vs. control; Figure 6, E-F), consistent with the occurrence of proteinuria. Importantly, this phenotype was also reproduced in *apol1* CRISPR/Cas9 larvae (Figure 9, E). Upon co-injection of WT APOL1 human mRNA, the increased dextran clearance in *apol1*-MO larvae was rescued significantly and fluorescence intensity returned to levels

indistinguishable from controls (n=28; p=7.75x10-4, MO vs. MO + mRNA; Figure 6, E-F).

Next, we evaluated the cellular organization and patterning of the developing glomerulus in the context of *apol1* suppression. We performed transmission electron microscopy (TEM) of ultrathin sections of zebrafish larvae at 5 dpf in WT and *apol1* morphants and mutants, with *myh9* morphants as a positive phenotypic control. In agreement with previous studies[123], *myh9* morphant larvae exhibit focal bulges and glomerular basement membrane (GBM) thickening in comparison to controls, as well as the presence of microvillus protrusions, a defining characteristic of proteinuria (Figure 10, G and Figure 11, B). Notably, *apol1*-MO injected larvae display a similar glomerular ultrastructure compared with *myh9* morphants. Naked patches of GBM are apparent throughout the glomerulus, indicative of extensive podocyte effacement (Figure 6, G-H and Figure 11, B). However, we did not observe GBM thickening as evident in *myh9*-MO injected larvae (Figure 6, G and Figure 11, B). In areas in which we did observe foot process formation, podocyte protrusions were irregular and inhibited slit diaphragm development (Figure 6, G-H and Figure 11, A). We also noted the formation of microvillus protrusion in the urinary space of *apol1* morphants. Similarly, *apol1*-CRISPR/CAS9 injected embryos display an aberrant glomerular ultrastructure, as

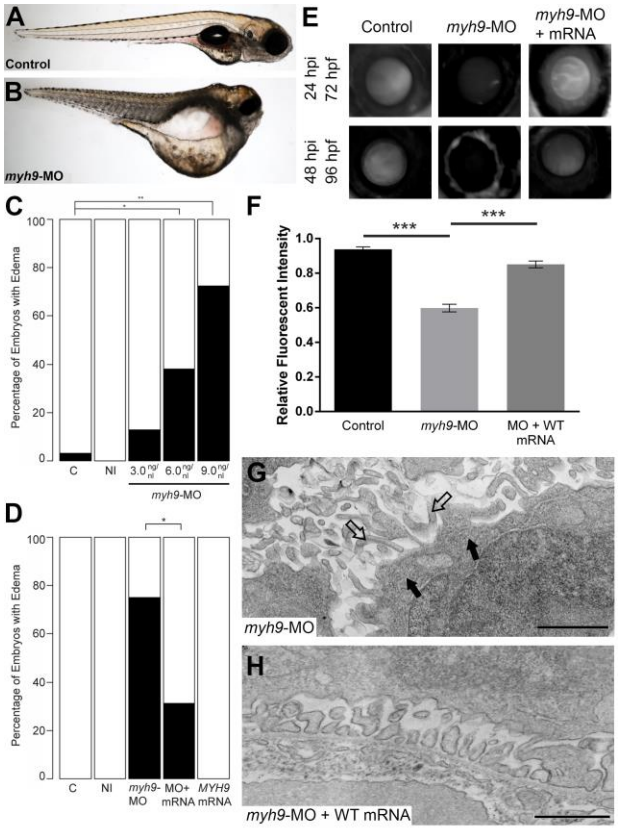

Figure 10: *myh9* suppression and complementation in developing zebrafish embryos.

We recapitulated data reported by Müller et al. for experimental comparison.[123] (A-B) Representative live images of sham-injected control and *myh9* morpholino (MO) injected larvae at 5 dpf. (C) Injection of increasing doses of *myh9* MO demonstrate dose-dependent effects when scored for generalized edema compared to control embryos at 5 dpf. (E-F) *myh9* morphants also display filtration defects indicated by significantly increased dextran clearance. (D-F) Co-injection of wild-type human *MYH9* mRNA (100pg/nl) significantly rescues edema formation and filtration defects observed in myh9 morphants. (G) As reported previously by Müller et al., *myh9* morphants display ultrastructure abnormalities, including glomerular basement membrane thickening and the presence of microvillus protrusions in the urinary space. (H) These ultrastructural defects are rescued upon co-injection of wild-type human *MYH9* mRNA (100pg). White bars, normal; black bars, edema; n = 49–70 and n = 13–29 embryos/injection batch for gross morphological scoring and glomerular filtration assays, respectively; *p<0.05; **p<0.01; **p<0.001; filled arrowheads, glomerular basement membrane; open arrowheads, microvillus protrusions.

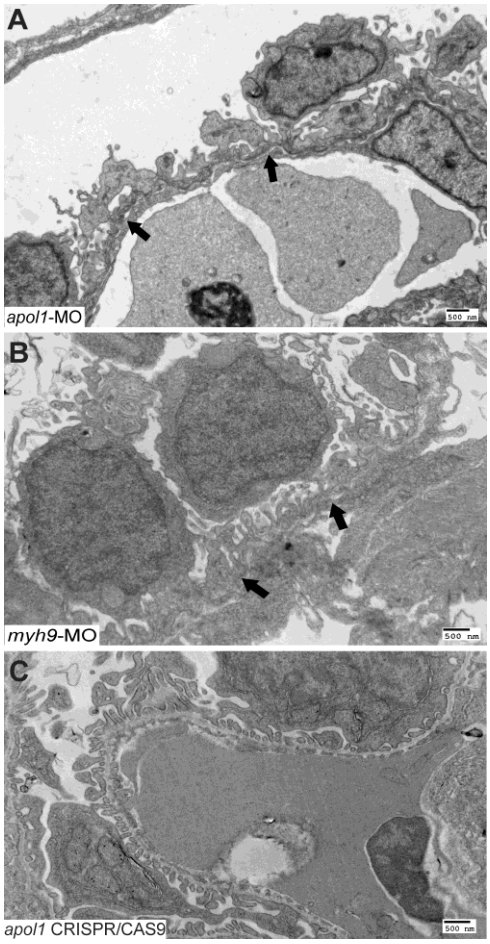

Figure 11: Further characterization of *apol1* and *myh9* morphant glomerular ultrastructure.

Transmission electron microscopy of zebrafish larval glomeruli injected with either (A) *apol1*-MO or (B) *myh9*-MO were imaged at 5 dpf using a low magnification (direct mag = 4400X) to characterize long stretches of the glomerular basement membrane (GBM). Comparatively, *apol1* and *myh9* morphants display similar abnormalities, including podocyte disorganization and effacement, as well as the presence of microvillus protrusions. However, *myh9* morphants display a thickened GBM that is not apparent in *apol1*-MO injected larvae, while *apol1* morphants appear to have a higher degree of podocyte effacement compared to *myh9* morphants. (C) Zebrafish larvae injected with *apol1* CRISPR/CAS9 display a similar glomerular ultrastructure compared to *apol1* morphants at 5 dpf. Filled arrowheads, glomerular basement membrane. Scale bar = 500nm.

evident by podocyte foot process effacement (Figure 9, F). Co-injection of orthologous WT human mRNA in *apol1* morphants rescued these glomerular ultrastructure defects (Figure 6, I). Together, these data represent compelling *in vivo* evidence implicating *APOL1* in renal function.

3.2.2 Complementation of zebrafish apol1 morphants with human *APOL1* risk alleles does not rescue kidney defects

Initial reports associating *APOL1* variants with kidney disease in African Americans identified two independent sequence variants, termed G1 and G2, which reside in a 10-kb region in the last exon of the gene.[92, 96, 148, 149] The G1 allele consists of two nonsynonymous coding variants in perfect LD, rs73885319 and rs60910145, while the G2 variant consists of a six base pair deletion that removes amino acids N388 and Y389 (~21% and ~13% allele frequency in African Americans, G1 and G2 respectively; (Figure 5, D). Therefore, we evaluated the ability of each of the G1 and G2 alleles to rescue *apol1*-MO injected zebrafish larvae. *APOL1* G1 (I384M/S342G) and G2 allelic constructs were generated from a WT *APOL1* human cDNA clone, transcribed, and co-injected with *apol1*-MO in zebrafish embryos (100pg/nl). Importantly, each *APOL1* allelic construct produces a stable protein detectable by immunoblotting when co-injected with *apol1*-MO (Figure 7). *apol1* morphants co-injected with either *APOL1* G1 (I384M/S342G) or G2 human mRNA did not display significant rescue of edema formation in developing embryos compared to *apol1*-MO injected embryos alone (Figure 12, A-B).

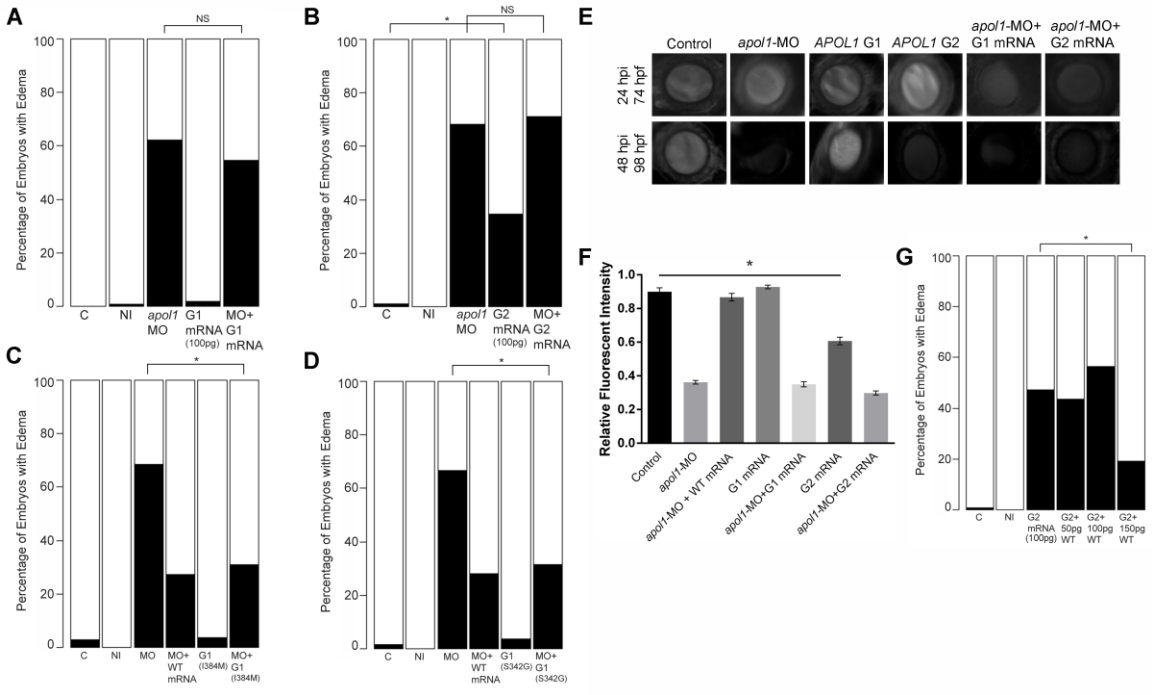

Figure 12: In vivo modeling of human *APOL1* variants associated with disease.

apol1 MO injected larvae were complemented with the respective human mRNA corresponding to *APOL1* G1 (S342G/I384M) (100pg/nl) and G2 (100pg/nl) risk variants and scored for edema formation at 5 dpf (n = 26–65 embryos/injection; repeated three times). (A, B) Neither risk variant of *APOL1* rescues significantly the edema phenotype observed in apol1 morphants. However, when human *APOL1* G2 mRNA was injected alone (B), a significant number of embryos develop edema compared to sham-injected controls, suggesting a possible dominant-negative effect of the G2 altered protein. (C, D) apol1 morpholino injected larvae were complemented with human mRNA corresponding to either (C) *APOL1* G1 I384M or (D) *APOL1* G1 S342G and scored for edema formation at 5 dpf (n = 48–93 embryos/injection; repeated two times). Each individual variant comprising *APOL1* G1 risk rescues significantly edema formation in apol1 morphant embryos, suggesting that both G1 variants must be present to confer loss of *APOL1* function. (E-F) *apol1* morphants co-injected with human *APOL1* G1 or G2 mRNA fail to rescue filtration defects as indicated by dextran clearance, while larvae injected with G2 mRNA alone display increased clearance over time. (G) Titration of G2 injected embryos with increasing concentrations of human WT *APOL1* mRNA show a significant reduction in edema formation of developing embryos at 5 dpf. White bars,normal; black bars, edema. C, sham-injected control; NI, non-injected control. *p <0.05.

In addition, we also co-injected each individual G1 variant (I384M and S342G) into apol1 morphant embryos. *APOL1* message encoding either p.I384M or p.S342G were individually able to rescue significantly the edema caused by *apol1* suppression (Figure 12, A-C) suggesting that the cis effect of both variants in the same haplotype is required to confer pathogenicity. When *APOL1* G2 mRNA was injected alone, a significant number of embryos developed edema in comparison to sham-injected controls (n = 52–63 embryos/injection; repeated three times; p = 0.012; Figure 12, B); no edema was observed with injection of 100pg APOL1 G1 mRNA alone (Figure 12, A). Additionally, dextran clearance assays demonstrated that neither *APOL1* G1 or G2 mRNA were able to rescue glomerular filtration defects caused by *apol1* suppression, while *APOL1* G2 mRNA injected alone caused significant filtration defects compared to controls (n = 12–21; p = 0.003, Control vs. G2 mRNA; Figure 12, E-F). Finally, when we injected embryos with *APOL1* G2 titrated with increasing concentrations of *APOL1* WT mRNA, we observed a significant reduction of edema formation in developing embryos (Figure 12, G) suggesting that this allele is conferring a dominant negative effect on protein function.

We also examined the glomerular ultrastructure of *apol1* morphants co-injected with either *APOL1* G1 or G2 human mRNA using TEM. However, we did not observe any noticeable improvement in glomerular ultrastructure abnormalities at 5 dpf (Figure 13, A-B).

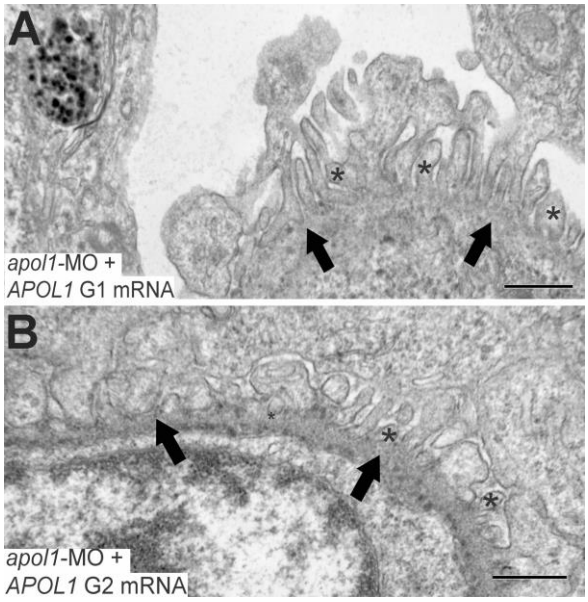

Figure 13: Glomerular ultrastructure of *apol1* morphants complemented with human risk alleles.

Transmission electron microscopy of zebrafish larval glomeruli imaged at 5 dpf. (A, B) *apol1* morphants complemented with risk alleles, G1 and G2 do not rescue the observed defects caused by *apol1* suppression, with naked patches of glomerular basement membrane and microvillus processes apparent. , microvillus protrusions; filled arrowheads, glomerular basement membrane. Scale bars, 500nm.

In concurrence with our observations of gross morphological defects, embryos injected with G2 mRNA alone also display glomerular aberrations and microvillus protrusions (Figure 14, A) similar to *myh9* and *apol1* morphants (Figure 6, H and Figure 11, A-C); no abnormalities were seen in larvae injected with G1 mRNA alone (Figure 14). These data provide direct evidence for a functional consequence of the human *APOL1* G1 and G2 risk alleles, and suggest that they confer loss-of-function and dominant negative effects, respectively.

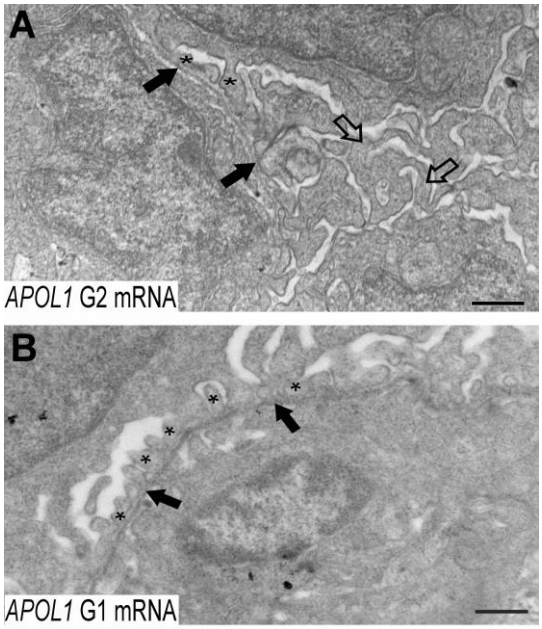

Figure 14: Overexpression of *APOL1* G2 causes glomerular defects similar to *myh9* larvae.

(A) Zebrafish embryos injected with *APOL1* G2 mRNA (100pg/nl) alone display glomerular aberrations similar to that of *myh9* suppressed larvae, with microvillus protrusions present (open arrowheads), although the glomerular basement membrane appears normal (filled arrowheads). Podocyte foot processes (* asterisk) are apparent, although sparsely present. (B) Embryos injected with *APOL1* G1 mRNA (100pg/nl) alone display normal glomerular ultrastructure. Scale bar, 500nm.

3.2.3 *myh9* and *apol1* interact under anemic stress to exacerbate nephropathy phenotypes

Although recent studies have provided statistical evidence implicating *APOL1* variation in nondiabetic nephropathies[148, 150, 151], *MYH9* risk variants are still associated with chronic kidney disease (CKD) in non-African American populations[152] and in sickle cell disease nephropathy.[96] As such, our group and others have hypothesized that these genes may be co-regulated to induce nephropathy risk; in fact, when we modeled glomerular filtration rate in sickle cell patients as a function of the previously reported

MYH9 risk haplotype and an *APOL1* recessive model, we observed a significant interaction between the two genes.[96] Therefore, we tested for functional interaction effects between *apol1* and *myh9* in zebrafish, an experimentally tractable model for investigating additive and synergistic effects.[153-157] First, we co-injected both *apol1*-MO and *myh9*-MO into embryos and we scored for gross morphological defects at 5 dpf. Under this co-suppression model, we observed no significant differences in edema formation when compared to batches injected with either MO alone (Figure 15, A), even when individual MO concentrations were reduced to subeffective doses (Figure 15, B). Next, we tested the possibility that suppression of either *apol1* or *myh9* in zebrafish could be rescued significantly by the co-injection of the reciprocal human mRNA. *myh9*-MO was co-injected with human *APOL1* WT mRNA (100pg/nl) and *apol1*-MO was co-injected with human *MYH9* WT mRNA (100pg/nl). However, we were unable to rescue the suppression phenotypes of either *apol1* or *myh9* with the human mRNA of the reciprocal gene (Figure 16), suggesting the two genes may work through different pathways to cause disease risk.

Our hypothesis for an interaction between *APOL1* and *MYH9* was based on data derived from SCD patients. Thus, we posited that *myh9* and *apol1* may only interact under additional biologic stress, such as anemia or hemolysis. Accumulating evidence suggests that both anemia and hemolysis, which are key features of SCD

pathophysiology, impact renal function; in particular, hemolysis appears to be associated with both microalbuminuria and hyperfiltration.[36, 158] While a zebrafish model

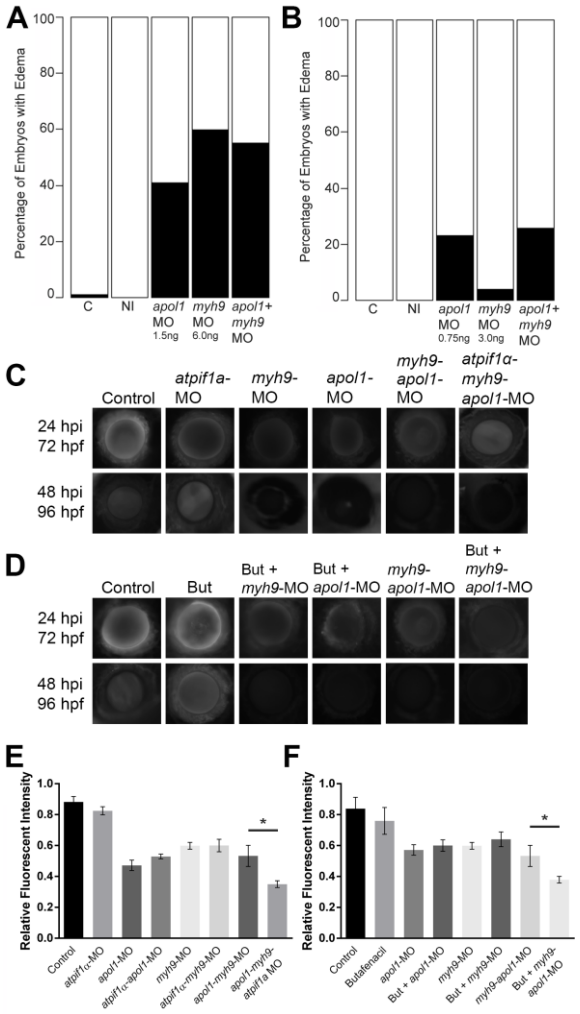

Figure 15: *apol1* interacts with *myh9* in an anemic context.

To test for epistatic effects of *apol1* and *myh9* in zebrafish, we first co-injected both *apol1*-MO (1.0ng/nl dose) and *myh9*-MO (6.0ng/nl dose) into zebrafish larvae and scored for edema formation at 5 dpf. (n = 39–89 embryos/injection; repeated three times). However, under this co-suppression model (A, B), we observed no significantly increased edema formation compared to each MO alone. We next tested for an interaction between *apol1* and *myh9* in the context of *atpif1a* suppression, predicting that the added stress of anemia would mimic our initial observations in sickle cell disease patients. 70kDa dextran-FITC conjugate was injected into the cardiac venous sinus of 48 hpf zebrafish larvae and fluorescence intensity in the eye vasculature was measured at 24 and 48 hours later. (C) Representative eye image series of zebrafish embryos for each injection

group show relatively stable or decreased fluorescence intensity over time. (E) Bar graphs summarize the changes observed for each injection group. Zebrafish embryos injected with all three MOs show a significant increase in dextran clearance from the vasculature compared to co-suppression of apol1 and myh9. (D, F) These data are reproduced using butafenacil induced anemia (0.195 μM in embryo media, treated at 48 hpf). Dextran values are in relative fluorescence intensity, mean ± SE. Control, sham-injected control (n = 19); *atpif1a* MO injected (n = 14); *apol1*-MO+*myh9*-MO (n = 12); *apol1*-MO+*myh9*-MO+*atpif1a*-MO (n = 11); Butafenacil (n = 48); But +*myh9*-MO+*apol1*-MO (n = 18). hpf, hours post-fertilization; hpi, hours post-injection. *p <0.001.

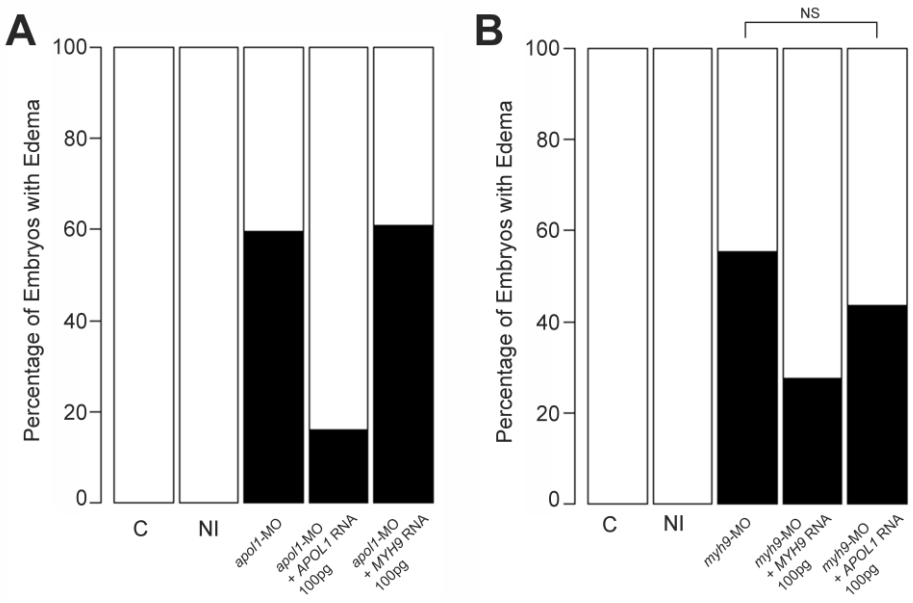

Figure 16: Complementation of *apol1* and *myh9* morphants with each respective reciprocal human wild-type mRNA.

(A) *apol1*-MO was co-injected with human WT *MYH9* mRNA (100pg/nl) and (B) *myh9*-MO was co-injected with human WT *APOL1* mRNA; embryos were scored for edema formation at 5 dpf (n = 25–66 embryos/injection for *apol1*-MO/*MYH9* RNA and n = 32–46 embryos/injection for *myh9*-MO/*APOL1* RNA); each repeated three times.

of SCD does not exist currently, suppression of ATPase inhibitory factor 1 (*atpif1α*), a mitochondrial protein, produces profound anemia in zebrafish by interfering with heme synthesis through decreased catalytic efficiency of ferrochelatase.[159] The resultant effect of low hemoglobin and hematocrit stresses the kidney because of the organ's high oxygen consumption. Consistent with the original report[159], we observed a dose-

dependent reduction in hemoglobin with increasing concentrations of the *atpif1a* MO (*atpif1α*-MO), as measured by o-dianisidine staining of whole MO-injected larvae at 4 dpf. Strikingly, we found a significantly more severe nephropathy phenotype in an anemic context as indicated by accelerated dextran clearance, with co-suppression of *apol1* and *myh9* under *atpif1α*-MO induced anemia (n = 12–19 embryos/injection; $p<0.001$ for *myh9*/*apol1* MOs vs. *myh9*/*apol1*/*atpif1a* MOs; Figure 15, C, E). Importantly, neither morphant alone resulted in a more severe phenotype under *atpif1α*-MO induced anemia (e.g. *myh9*-MO vs. *myh9*-atpif1α-MO; $p = 0.78$; or *apol1*-MO vs. *apol1-atpif1α*-MO; $p = 0.90$; Figure 15, E). Furthermore, these observations were reproducible using an independent and non-genetic induction of anemia. Butafenacil, an inhibitor of protoporphyrinogen oxidase, causes loss of hemoglobin following exposure during early zebrafish development.[160] In a butafenacil-induced anemic context (0.195 μM treatment at 48 hpf), we observed a similar effect upon co-suppression of *apol1* and *myh9* (n = 17–23 embryos/injection; $p<0.001$ for *myh9*/*apol1* MOs vs. *myh9*/*apol1* + 0.195 μM butafenacil; Figure 15, D and F).

3.2.4 *APOL1* G2 (del:N388Y389) modulates *myh9* expression *in vivo*

To dissect further the possible genetic interactions between *myh9* and *apol1*, we tested whether suppression of endogenous *apol1* or ectopic expression of mutant human *APOL1* could alter expression of *myh9* in zebrafish embryos. We monitored *myh9* expression in zebrafish larvae using qRT- PCR in the context of *apol1* suppression, and

G1 or G2 expression, as well as *apol1*/*APOL1* modulation in conditions of anemia induced by *atpif1α*-MO injection at 5 dpf (Figure 17, A) and 3 dpf (Figure 17, B).

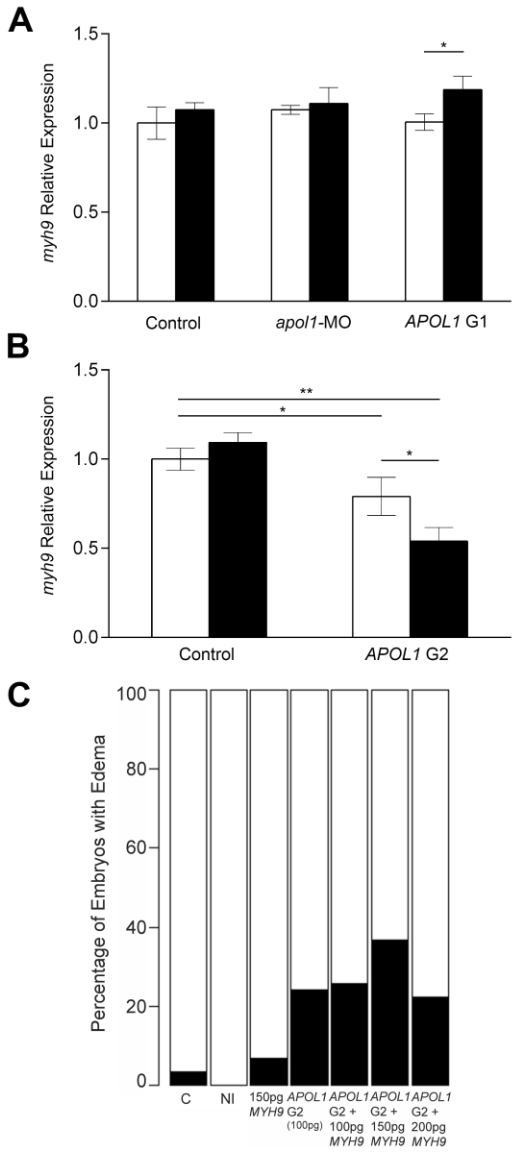

Figure 17: *myh9* expression in the context of *apol1*/*APOL1* modulation.

Zebrafish embryos were injected with either *apol1*-MO (1.0ng/nl dose), *APOL1* G1 (S342G:I384M) mRNA (100pg), or *APOL1* G2 (100pg) mRNA alone, in the absence (white bars) or presence (black bars) of *atpif1α*-MO. Total RNA at 5 dpf or 3 dpf (*APOL1* G2/*atpif1α*-MO embryos did not survive to 5 dpf) was extracted and reverse-transcribed with random primers to obtain whole-embryo cDNA. *myh9* expression was determined by quantitative real-time PCR and relative

expression was calculated against *actb1*. (A) *apol1*-MO injected embryos do not display any significant changes in *myh9* expression compared to sham-injected control embryos. Additionally, *APOL1* G1 expression does not alter myh9 expression alone, however, under *atpif1α*-induced anemia, we observe an increase in myh9 expression. (B) *APOL1* G2 expression results in a significant decrease in *myh9* expression compared to sham-injected control embryos, suggesting that the altered APOL1 protein may regulate *myh9* in vivo. (C) Coinjection of *APOL1* G2 (100pg) and human WT *MYH9* (n = 31 –60; repeated two times), does not rescue edema formation caused by *APOL1* G2 expression in 5 dpf larvae, suggesting that the interaction between *APOL1* G2 and MYH9 may be indirect. Relative expression values are mean ± SE in triplicate with two biological replicates. * = $p < 0.05$; ** = $p < 0.01$.

We observed a significant decrease in *myh9* expression when zebrafish embryos were injected with the proposed dominant-negative *APOL1* G2 allele alone (21% reduction; $p = 0.043$; Figure 17, B), suggesting that the mutant protein may be suppressing *myh9*, either directly or indirectly, to induce nephropathy. Furthermore, zebrafish embryos co-injected with *APOL1* G2 mRNA and *atpif1α*-MO display an even greater reduction in *myh9* expression compared to controls (46% reduction; $p = 0.0013$; Figure 17, B), and a significant reduction of *myh9* expression compared to *APOL1* G2 mRNA alone ($p = 0.0297$; Figure 17, B), suggesting that the altered APOL1 (p.Asn388_Tyr389del) protein has a more pronounced effect on *myh9* expression in the context of anemic stress. We also observed a significant increase in *myh9* expression in *APOL1* G1/*atpif1α*-MO vs. *APOL1* G1 injected embryos (Figure 17, A), however, neither of these conditions induced nephropathy. To determine whether this effect was specific to *myh9* or was a general effect on transcripts expressed in the glomerulus, we also assessed expression levels of other nephropathy-associated genes during *apol1*/*APOL1* modulation and *atpif1α* induced anemia. We observed no significant differences in expression of genes

implicated in familial focal segmented glomerulosclerosis, including *anln*[161], *trpc6b*[162], and *wt1a*[163] upon *apol1*/*APOL1* modulation (Figure 18), suggesting that *APOL1* G2 regulation may be specific to *myh9*.

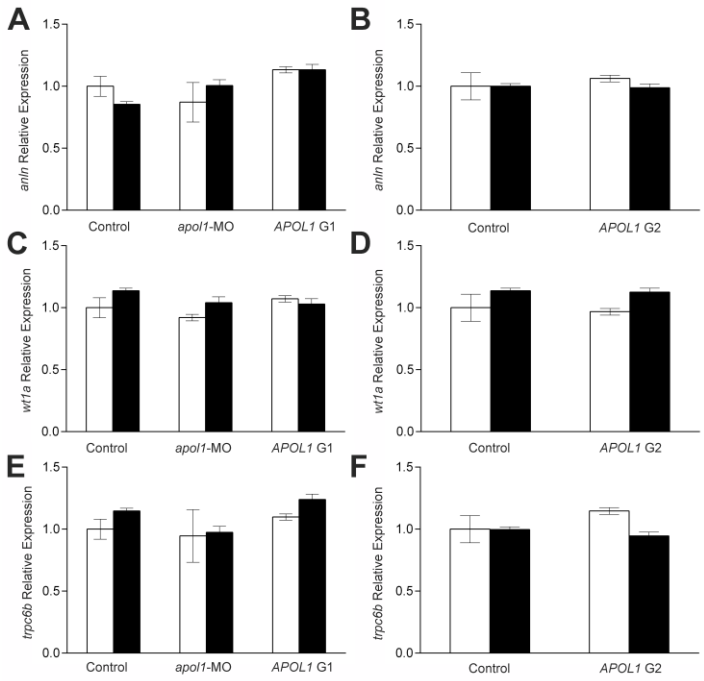

Figure 18: *apol1*/*APOL1* modulation effect on causal familial Focal Segmental Glomerulosclerosis (FSGS) genes.

Zebrafish embryos were injected with either *apol1*-MO (1.0ng/nl dose), *APOL1* G1 (S342G:I384M) mRNA (100pg), or *APOL1* G2 (100pg) mRNA alone, in the absence (white bars) or presence (black bars) of *atpif1α* -MO. Tot al RNA at 5 dpf or 3 dpf (*APOL1* G2/*atpif1α*-MO embryos did not survive to 5 dpf) was extracted and reverse-transcribed with random primers to obtain whole-embryo cDNA. (A-B) *anln*, (C-D), *wt1a*, (E-F) or *trpc6b* expression was determined by quantitative real-time PCR and relative expression was calculated against *actb1*. We observed no significant differences in expression in any of the FSGS-associated genes tested under *apol1*/*APOL1* modulation, suggesting that *APOL1* G2 regulation may be specific to *myh9*. White bars = normal; black bars = *atpif1α*-induced anemia. Relative expression values are mean ± SE in triplicate with two biological replicates.

Based on the observations that *APOL1* G2 expression has the ability to decrease *myh9* expression in vivo, we next attempted to rescue *APOL1* G2 defects by co-injecting

human WT *MYH9* mRNA. We injected a constant amount *ofAPOL1* G2-encoding message (100pg) with increasing amounts of human *MYH9* mRNA (100pg, 150pg, and 200pg) and scored larvae live for generalized edema at 5dpf. However, we did not observe a significant reduction of edema in *APOL1* G2/*MYH9* co-injected embryos (Figure 17, C), suggesting that compensation with *MYH9* message alone is not sufficient to account for the deleterious effects of the G2 variant, possibly because *APOL1* G2 has a trans effect on other loci in the genome or is acting to perturb cellular pathways.[138]

3.3 Discussion

In recent years, multiple lines of statistical evidence have implicated the *MYH9*/*APOL1* locus on chromosome 22q12.3 with nondiabetic end-stage renal disease, focal segmental glomerulosclerosis, HIV-associated nephropathy, lupus nephritis, SCDN, and diabetic nephropathy in patients of recent African ancestry and European Americans.[80, 81, 92, 96, 148, 149 150, 152, 164-166] Additionally, *APOL1* has been associated with an increased burden of cardiovascular disease in African Americans participating in the Jackson Heart Study.[167] Compelling statistical evidence in human cohorts points to the G1 and G2 alleles of *APOL1*, rather than *MYH9* variation, as the most likely contributors to nephropathy risk. Nonetheless, functional studies of the *MYH9* locus provide biological evidence for its role in the kidney, including perturbed glomerular development in myh9 morphant zebrafish.[121-123, 136] Here, we have identified a functional ortholog of human *APOL1* in zebrafish and, using transient genetic manipulation,

provide functional evidence demonstrating *apol1* involvement in both kidney development and filtration.

Although the human *APOL* gene cluster has undergone recent natural selection in primates[125, 142], we report the identification of a functional *APOL1* ortholog in the zebrafish genome and its implication in renal function. Specific detection of the zebrafish apol1 protein product with the human *APOL1* antibody, rescue of kidney defects in apol1 morphant embryos with human *APOL1* mRNA, as well as recapitulation of renal phenotypes with an *apol1*-CRISPR/CAS9 F0 mutant, provide evidence that zebrafish *apol1* is indeed functionally relevant to its human ortholog with respect to its role in the glomerulus. Furthermore, no other human mRNA in the human apolipoprotein L family ameliorated kidney defects induced by *apol1* knockdown, supporting further its functional orthology to human *APOL1*. Nonetheless, it is unclear whether the zebrafish APOL1 protein serves all functions of its human counterpart, especially given the lack of a secretory domain in the zebrafish APOL1 peptide (Figure 5, A).

Suppression and genome-editing of *apol1* in zebrafish and three independent phenotypic scoring paradigms support a role for *apol1* in nephropathy; we observed severe edema formation with concomitant glomerular filtration defects and severe podocyte loss. Complementation of *apol1* suppression with *APOL1* CKD risk alleles (G1 and G2) failed to ameliorate these observed defects. Notably, complementation of each individual variant of the G1 haplotype (I384M and S342G) rescued significantly

nephropathy phenotypes caused by *apol1* suppression, suggesting that both variants must be present in cis to confer risk. This is concordant with initial reports on the lytic potential of APOL1 recombinant proteins on T. b. rhodesiense, in which *APOL1* variants with either S342G or I384M alone were less lytic than if both were present together.[92]

Strikingly, injection of human *APOL1* G2 mRNA alone resulted in significant edema formation in 5 dpf zebrafish larvae as well as perturbed glomerular filtration and ultrastructural defects. Our expression data suggest that this could arise from *myh9* suppression induced by the altered APOL1 protein harboring the G2 variant. The G2 deletion lies in the SRA-binding domain of APOL1 (Figure 5, B and D). Therefore it is plausible that disruption of this region of the protein may either prohibit proper binding of APOL1 to its usual partners, or perhaps permit new interactions that induce nephropathy. Further studies are needed to elucidate the functional impacts of the altered APOL1 protein to nephropathy. We also report for the first time functional evidence of a genetic interaction between *myh9* and *apol1*. Intriguingly, this interaction was only observed in the presence of anemic stress, consistent with our previous genetic association findings in human SCD patients.[96]

An immediate question remains regarding the mechanism by which *apol1* suppression is inducing kidney injury. Early studies revealed *APOL1* mRNA expression in the placenta, lung, and liver, with specific cell-type expression found in endothelial cells and possibly macrophages.[125] More recent studies, however, have characterized the

cellular localization of APOL1 in human kidney sections to podocytes, proximal tubules, and arteriolar endothelial cells.[134] These data are consistent with our observation of *apol1* morphants and mutants exhibiting extensive podocyte loss and suggest that *apol1* is necessary for the development and/or maintenance of glomerular podocytes. Interestingly, it has been shown that *APOL1* may cause toxic renal effects through programmed cell death pathways leading to glomerulosclerosis.[168, 169] Thus, *apol1* suppression could dysregulate autophagic pathways, causing podocyte malformation, thereby promoting the susceptibility of the pronephros to glomerular injury.

Initial studies implicating *MYH9* in nondiabetic nephropathy failed to identify coding variants associated with renal outcome[80, 81], and since the nearby nonsynonymous variants identified in *APOL1* provided stronger statistical association[92, 96, 148], it was hypothesized that *APOL1* variation represents the true attribution to renal disease risk. In fact, it has been shown in multiple studies that controlling for the *APOL1* risk alleles (G1-G2) attenuates significantly the effect of *MYH9* SNPs.[92, 150] However, recent reports still demonstrate statistical association of *MYH9* in nondiabetic nephropathy[96, 152] and previous *in vivo* modeling studies provide further evidence for the role of *Myh9* in glomerular development and glomerulosclerosis.[121, 123, 136] As such, our group and others have postulated that complex genetic models may exist in this region, including the possibility of *MYH9-APOL1* gene interaction.[96, 149] Our observation of exacerbated glomerular filtration in the context of anemic stress provides biological evidence in

support of this hypothesis. Because knockdown of each of *myh9* and *apol1* independently impairs proper pronephric development and filtration, it is plausible that their encoded proteins are functioning in separate pathways to induce kidney dysfunction. However, these effects only appear to become additive under an additional stress (anemia). The associated variants alone may not be sufficient to induce nephropathy progression, while under low hemoglobin and hematocrit levels, additive effects between *MYH9* and *APOL1* may become apparent and result in a more drastic reduction in renal function, along with the observed significantly high early mortality rates among SCD nephropathy patients.[4, 16, 36, 48]

Furthermore, we provide evidence suggesting that the functional consequences of *APOL1* variation may not be acting in a strictly recessive manner as had been previously suggested.[92, 96, 148, 170] Our data demonstrate that *APOL1* G1 (I384M/S342G) confers loss of proper *APOL1* function in the developing zebrafish kidney, while *APOL1* G2 is acting in a dominant-negative manner to induce nephropathy, possibly through suppression of *myh9*. These data indicate that the risk conferred by the *APOL1*/*MYH9* locus is likely to be governed by a more complex model than recessive patterning as suggested previously.

In summary, our study demonstrates the essential role of both *apol1* and *myh9* in the development of the pronephric glomerulus and proper renal filtration in zebrafish. We report comprehensive *in vivo* causal evidence of *apol1* involvement in kidney decline,

and we provide the first *in vivo* evidence of a potential dominant-negative effect of the *APOL1* G2 allele. Further, we have shown that the presence of the G2 allele decreases significantly the expression of *myh9*. Similar to the common haplotype on 10q26 that influences age-related macular degeneration underscored by complex regulatory events of neighboring genes *ARMS2* and *HTRA1*, our data highlight further the importance of comprehensive evaluation of functional consequences at a susceptibility locus.[171] Taken together, these data provide essential biological insight into the mechanisms by which *MYH9* and *APOL1* confer disease risk and progression in human nondiabetic nephropathies.

4. Identification and Functional Evaluation of Novel SCDN Candidates

4.1 Novel genetic variation associated with renal outcomes in sickle cell disease using GWAS

As mentioned in previous chapters, sickle cell disease clinical sequelae and survival varies greatly and depends on both genetic and environmental factors. Notably, candidate gene studies[96, 172-182] and GWAS[178, 183-185] have achieved modest success in identifying genetic risk for end-organ damage in SCD patients. However, knowledge regarding the genetics underlying renal function and disease progression among SCD patients remains limited. As shown in the above chapters, we have identified the *MYH9*/*APOL1* locus on chromosome 22q12.3 to be significantly associated with proteinuria in SCD and demonstrated that an interaction between the two genes predicts

GFR.[96] Specifically, in Chapter 3, we discovered a functional role for *APOL1* and provide critical data that *APOL1* and *MYH9* interact genetically, particularly under anemic stress. However, variants at the *MYH9*/*APOL1* locus appear to only explain a part of the disease risk, as the frequency of proteinuria associated with particular SNPs ranged from 35%-50%.[96] This suggests additional genetic factors may be contributing to renal outcome in SCD patients. As such, we performed an unbiased interrogation of the genome (GWAS) in order to uncover putative new nephropathy genes for genetic evaluation.

Glomerular filtration rate (GFR) is a known marker of kidney decline in SCD and was first assessed in our GWAS analysis. 463 patients with complete data were included in the analysis (see Table 5). The patient cohort was collected as part of a multicenter study of outcome-modifying genes in SCD and participating institutions included

Table 5: Patient characteristics included in renal outcome GWAS

	Number of Patients	Percent / Mean (SD)
Age at enrollment	463	34.22 (12.30)
Percent Male	210	45.36
Hb Genotype		
HbSS	389	84.93
HbSβ^0	16	3.49
HbSβ^+	13	2.84
HbSC	40	8.73
GFR	463	152.12 (67.26)
GFR < 90 ml/min/1.73m^2	81	17.49
Proteinuria	114	25.68

sickle cell centers at Duke University, University of North Carolina at Chapel Hill, Emory University, and East Carolina University. GFR was estimated using the 'Modification of Diet in Renal Disease' (MDRD) study definition[99] and dichotomized at the clinically relevant threshold of 90 ml/min/1.73min^2 (CKD stage 2). Genotyping was performed using the Illumina Human610-Quad BeadChip and a global reference panel from the 1000 Genomes project was used to impute genotypes not covered on the GWAS chip. Samples were pre-phased with SHAPEIT[186] and genotypes inferred using IMPUTE2[187]. After data cleaning and removal of SNPs with minor allele frequency (MAF) < 5%, 3,021,990 SNPs were available for analysis. Principal component (PC) analysis was performed to obtain measures of population stratification in our data set using EIGENSOFT[188]. Logistic regression was utilized to test for association between each SNP and dichotomized GFR (CKD stage 2 or greater), controlling for genome-wide PCs using PLINK[189]. False discovery rate (FDR) p-values were generated using PROC MULTTEST in SAS.

Using the imputed data from this cohort containing over 3 million SNPs, we identified 38 SNPs nominally associated (p-value < 5×10^{-5}) with CKD stage 2 or greater. The most significantly associated SNPs resided in two genes, xylosyltransferase I (*XYLT1*) and solute carrier family 2, member 9 (*SLC2A9)* ($p=6.8\times10^{-6}$ and $p=3.8\times10^{-5}$, respectively). Of note, *XYLT1* has been previously implicated in a cohort of 912 diabetic nephropathy patients and specifically associated with serum creatinine levels, a measure

taken into account in the MDRD GFR study definition. *SLC2A9* (also known as glucose transporter 9, *GLUT9*) has also been previously associated with kidney disease, including exercise-induced kidney failure[190-192] and CKD progression.[193] In addition to testing for association with CKD stage and GFR, we also assessed variation in relation to proteinuria as a renal outcome in this SCD cohort. Here, the occurrence of proteinuria was defined by dipstick analysis and classified as either positive or negative for the presence of albumin in urine. 26 SNPs were nominally associated with proteinuria in this SCD cohort. The most significant SNP resides in crystalline, lambda-1 (*CRYL1*) ($p=9.4x10^{-7}$), a gene predominantly studied in the context of hepatoceullar carcinoma but is highly expressed in the human kidney.[194]

As GWAS chips only capture common variation, it is often the case that associated SNPs fall in intergenic or noncoding regions of the genome. Indeed, this was the result in our analysis, and although we found associations with both GFR and proteinuria, all significant SNPs were located in noncoding regions of each gene. As such, we next analyzed a subset of the patient samples from the Duke cohort (n=429) on the Illumina HumanExome Beadchip, which focuses on functional and exonic genomic content. This platform is complementary to traditional GWAS chips, as it contains only coding variation that is generally less frequent among populations. Using the gene-based test SKAT-O to identify genes associated with proteinuria in the Duke cohort, we found three genes that met an FDR $q < 0.2$: family with sequence similarity 114 Member

A2 (*FAM114A2*), keratocan (*KERA*), and leucine-rich repeat-containing protein 6 (*LRRC6*). Of these three genes, only *LRRC6* has a previous association with a kidney phenotype. *LRRC6* mutations cause primary ciliary dyskinesia, and was described originally in zebrafish as the seahorse mutant, in which loss of function results in cystic kidneys.[195, 196] Although not FDR significant, *XYLT1* was nominally associated with proteinuria in the gene-based rare variant analysis ($p=8x10^{-4}$), providing further support for the GWAS association. We also performed the SKAT-O test with GFR as the outcome, again dichotomizing at CKD stage 2 (90 ml/min/1.73min^2). The only gene meeting FDR significance ($q< 0.2$) was acyl-CoA synthetase family, member 3 (*ACSF3*), a gene previously associated with combined malonic and methylmalonic aciduria,[197] and highly expressed in the kidney.[198] Results from these GWAS are summarized in Table 6.

Table 6: Duke SCD renal outcome GWAS and ExomeChip association results

Gene	Chr Position	Associated Outcome	Genotype Platform	P-value
XYLT1	16p12.3	Proteinuria / GFR	GWAS / ExomeChip	$6.8x10^{-6}$ / $8.0x10^{-4}$
CRYL1	13q12.11	Proteinuria	GWAS	$9.4x10^{-7}$
SLC2A9	4p16.1	GFR	GWAS	$3.8x10^{-5}$
ACSF3	16q24.3	GFR	ExomeChip	$7.2x10^{-4}$
FAM114A2	5q33.2	Proteinuria	ExomeChip	$4.0x10^{-6}$
KERA	12q21.33	Proteinuria	ExomeChip	$1.3x10^{-5}$
LRRC6	8q24.22	Proteinuria	ExomeChip	$1.0x10^{-5}$

4.2 Functional evaluation of novel SCDN candidates

In order to bolster the evidence that these genes are relevant to SCD nephropathy, we utilized a zebrafish modeling platform to assay involvement in renal function. Using reciprocal BLAST searches against the *D. rerio* and *H. sapiens* genomes, we identified the zebrafish ortholog(s) to our newly-identified candidate genes (Table 7). When possible, we designed a splice-blocking (sb) morpholino (MO) rather than a translation-blocking (tb) MO to knockdown the expression of the candidate ortholog (Table 7). Each MO was injected at the 1-4 cell stage and the effect and efficiency of each splice-blocking MO was evaluated by PCR amplification followed by sequencing of the target region in each gene. We then tested the phenotypic effect of gene knockdown of each of our candidate genes by first assessing the formation of gross organismal defects.

Table 7: Zebrafish ortholog conservation and MO design of SCDN candidates

Human Gene	Zebrafish Ortholog	Nucleotide Conservation (%)	MO Target	Knockdown Effect
ACSF3	*acsf3*	56.0	exon 3 (sb)	exon 3 deletion
CRYL1	*cryl1*	55.0	exon 3 (sb)	exon 3 deletion
FAM114A2	*fam114a2*	49.0	exon 5 (sb)	exon 5 truncation
KERA	*kera*	56.0	ATG (tb)	translation inhibition
LRRC6	*lrrc6*	55.0	ATG (tb)	translation inhibition
SLC2A9	*slc2a15a*	45.0	exon 4 (sb)	exon 4 truncation
	slc2a15b	48.0	exon 3 and 5 (sb)	inefficient MO
XYLT1	*xylt1*	65.0	exon 3 (sb)	exon 3 deletion

Of note, we were not able to assess the effect of *SLC2A9* (*slc2a15a, slc2a15b*) knockdown due a duplication event in the zebrafish genome resulting in inefficient MO design. General edema or cardiac edema was scored at 5 days-post-fertilization (dpf), a phenotype we have previously correlated with reduced kidney function in developing embryos (see Chapter 3). Knockdown of *xylt1, cryl1, fam114a2,* and *lrrc6* resulted in a dose-responsive development of pericardial edema at 5 dpf, suggesting that these genes potentially impact renal function (Figure 19). Suppression of either *acsf3* or *kera* did not produce a detectable morphological defect relevant to the kidney. Notably, *KERA* did not have literature evidence supporting CKD involvement or kidney function.

Although we and others have shown a phenotypic correlation between pericardial edema formation and kidney dysfunction in developing zebrafish embryos, we nonetheless sought to test the effect of gene knockdown in an assay more specific to kidney function. Using a previously generated double transgenic line (pod::NTR-mCherry/VDBP-GFP),[144] in which mCherry is specifically expressed in podocytes under control of the zebrafish *podocin* promoter, along with the bacterial nitroreductase (NTR), and a vitamin D-binding protein-GFP fusion (VDBP; the zebrafish homolog to albumin) is expressed under the control of a liver-specific fatty acid-binding protein (*l-fabp*) promoter, allowing VDBP-GFP to be produced in the liver and excreted into the bloodstream. As such, this model not only allows the visualization of podocyte integrity, but also provides the ability to test glomerular filtration barrier (GFB) function by

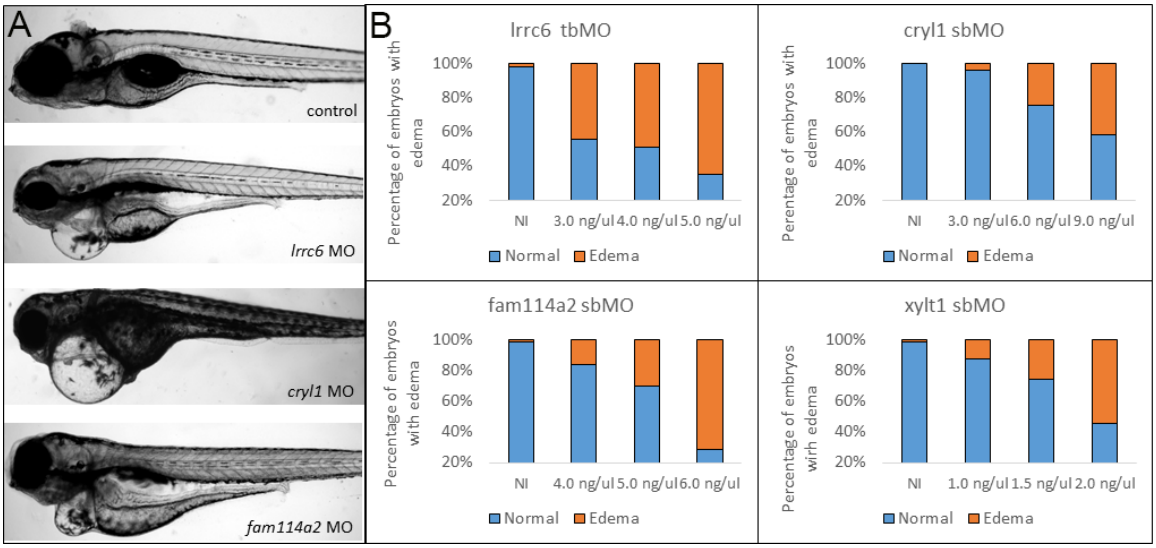

Figure 19: *In vivo* assay of SCD nephropathy candidates identified by GWAS.

Wild-type zebrafish embryos were injected with increasing doses of each respective morpholino (MO), and scored live for edema at 5 days-post-fertilization (dpf). (A) Representative live images of edema formation in *lrrc6*, *cryl1*, and *fam114a2* morphants at 5 dpf. (B) Quantification of edema formation in *lrrc6*, *cryl1*, *xylt1* and *fam114a2* morphants (n=54-87, repeated.

quantitatively measuring GFP accumulation in embryo medium using ELISA. As proof-of-principle, we first recapitulated data to test whether VDBP-GFP will leak through the GFB of the pronephros in pod::NTR-mCherry/l-fabp::VDBP-GFP double transgenic larvae. 3 dpf embryos were placed into 12-well plates (20 embryos per well) and treated with 5mM or 10mM metronidazole (MTZ). Embryos were treated with 0.1% DMSO as a control. After 24 hours, 100µl of embryo medium was assayed using the GFP ELISA kit (Cell Biolabs, San Diego, CA) following the manufacturer's protocol. Each assay was run in duplicate. Treatment with MTZ, which induces toxicity specifically in podocytes due to the NTR cassette, shows significant accumulation of VDBP-GFP in embryo media compared to controls (Figure 20). Given the validation of this assay, we next tested the

effect of gene knockdown of our SCDN candidates on zebrafish GFB function. Importantly, we also co-injected human mRNA corresponding to each candidate gene to test for functional specificity between the human and zebrafish orthologues. Among our SCDN candidate genes identified through GWAS, *lrrc6*, *acsf3*, *xylt1*, *fam114a2*, and *cryl1* show significant accumulation of VDBP-GFP in the surrounding media upon MO-knockdown of each respective gene (Figure 21). Of note, co-injection of human wild-type mRNA corresponding to *LRRC6*, *ACSF3*, *CRYL1* and *XYLT1* rescued significantly GFP leakage into the media as quantified by ELISA (Figure 21). Human *FAM114A2* mRNA, however, was not able to rescue GFB defects in morphants, suggesting that the human protein may have a divergent function compared to its zebrafish ortholog (Figure 21). Although this gene may still be a likely candidate for SCDN progression we determined that, using our current zebrafish assay, we could not conclude functional relevance to kidney decline. In summary, using GWAS targeting both common and rare variation in a clinically well-characterized adult SCD cohort, we have identified putative novel candidates for SCDN risk. Furthermore, we provide functional relevance to a subset of these candidates using a zebrafish model specific to GFB integrity.

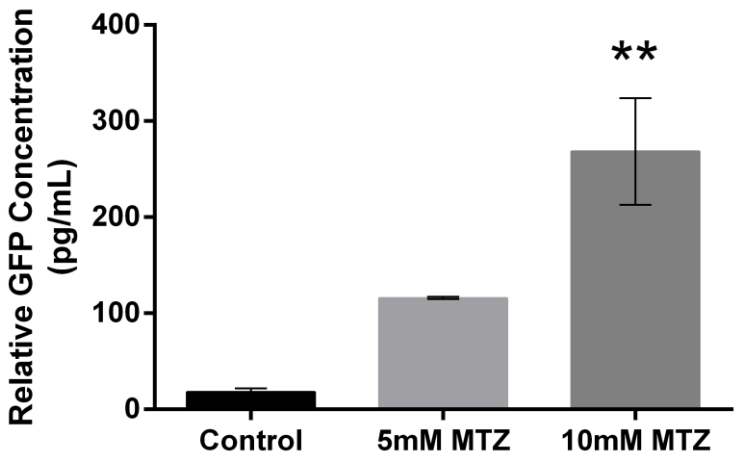

Figure 20: Assessment of glomerular filtration barrier function in a NTR inducible zebrafish model.

pod::NTR-mCherry/l-fabp::VDBP-GFP double transgenic zebrafish were treated with metronidazole (MTZ) at 3 days-post-fertilization. MTZ is converted to a cytotoxin in podocytes by nitroreductase (NTR), inducing cell death and breakdown of glomerular filtration barrier (GFB) integrity. Vitamin D-binding protein (zebrafish albumin) subsequently leaks out into the surrounding media and is quantitatively assessed using ELISA against GFP. At 10mM treatment of MTZ, pod::NTR-mCherry/l-fabp::VDBP-GFP embryos display significant accumulation of GFP compared to controls. **$p<0.01$. n=20 embryos/condition, ran in duplicate. Repeated two times.

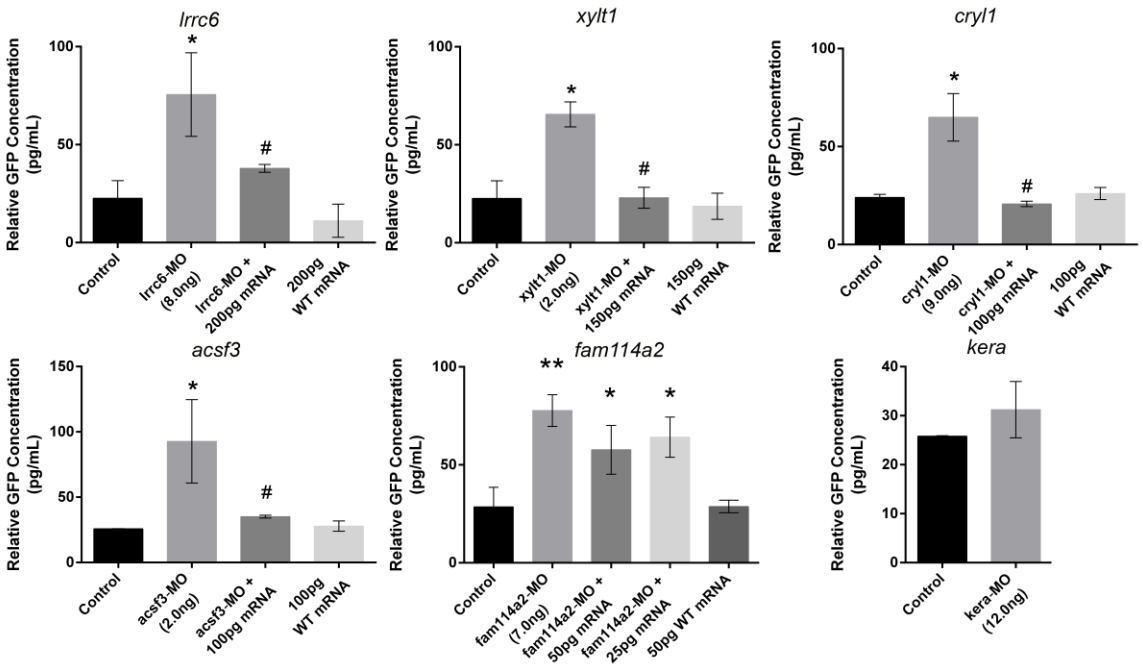

Figure 21: Impact of SCDN candidate gene knockdown on glomerular filtration barrier function in zebrafish larvae

pod::NTR-mCherry/l-fabp::VDBP-GFP double transgenic zebrafish embryos were injected at the 1-4 cell stage with either morpholino (MO) alone, co-injected with MO and human mRNA, or human mRNA alone. At 3 days-post-fertilization (dpf), 20 embryos per condition (in replicate) were place in separate wells. After 24 hours, 100µl of embryo medium was assayed with a GFP ELISA kit and relative GFP concentration was quantified, which corresponds to the amount of VDBP (albumin) present. Experiments were repeated 2-3 times. *p<0.05, **p<0.01 compared to controls. #p<0.05 compared to MO alone.

4.3 Deep sequencing of SCDN candidates

GWAS and candidate gene discoveries in isolation have been hampered by the inability to mechanistically attribute an associated locus to disease. To address this problem, we have attempted to layer our genetic association studies with functional annotation using our zebrafish model system and have identified novel candidates that

may incur risk for SCDN. However, these results only provide evidence for a role of each individual transcript as a whole to kidney function. Thus, the identification of putative variants within these genes and their role in kidney function would be more informative to patient populations and for SCDN etiology. As such, we performed a comprehensive study using next-generation sequencing (NGS) to interrogate our SCDN candidates in the Duke cohort of adult SCD patients (n=549). In short, amplicons were designed to target exonic coding regions and intron-exon boundaries of seven candidates genes previously identified through GWAS (see Table 7). Target regions were then amplified and Ion Torrent libraries were constructed. The Ion Personal Genome Machine (PGM) was used to generate deep sequencing reads in an attempt to identify enrichment for rare variants correlated with SCDN. Across all samples we achieved a mean coverage of 262x, giving us adequate read depth to identify rare variants present in our patient samples that are associated with renal outcome in SCD. Read alignments and variant calling was performed using our previously established NGS analysis pipeline.[199, 200] We identified significant enrichment of rare variants associated (using SKAT-O) with either proteinuria or GFR in several of our candidates from GWAS, including *ACSF3*, *FAM114A2*, *LRRC6*, and *KERA* (Table 8). Importantly, we confirmed all non-synonymous coding variants identified by PGM deep sequencing with Sanger sequencing to eliminate any false-positive results caused by NGS

technology. All SNPs used in the association analysis had a MAF <5%, used SKAT weights[201] and were coding variants.

Table 8: Ion Torrent PGM deep sequencing results for SCDN candidates

P-values represent gene-based SKAT-O test for association. N Marker describe the number of identified SNPs used in the analysis.

Gene Name	P-value (prot)	P-value (GFR)	N Marker (prot)	N Marker (GFR)
SLC2A9	0.805	0.870	13	11
FAM114A2	0.030	0.372	11	11
LRRC6	0.001	0.071	12	12
KERA	0.002	0.235	4	4
CRYL1	0.261	0.743	6	7
XYLT1	0.574	0.434	24	23
ACSF3	0.840	0.018	8	7

These data provide further support for the role of these candidate genes in SCDN pathology. Of note, 3/7 candidates (*SLC2A9, CRYL1,* and *XYLT1*) were not found to be significant in this SKAT-O test, contrary to our results from GWAS. This could represent a distinction between common and rare variant association with nephropathy outcome in SCD, however, further dissection is needed. Importantly, we have identified putative functional rare variants that may be relevant to kidney decline in SCD. The zebrafish model has proven to be a powerful tool to evaluate the *in vivo* function of missense variants identified in human populations.[155, 202-207] Using this model, we will be able to functionally examine these alleles in zebrafish to determine benign, null, or gain of function variants present in SCDN patients.

4.4 Meta-analysis and pleiotropy in SCDN

Although we have identified possible novel variation associated with nephropathy outcomes in SCD patients through GWAS, the effect size due to these alleles is relative small. In fact, genetic effects due to common variation are generally slight and so the detection of signals often requires much larger samples sizes.[208-211] As a single GWAS can be underpowered, performing a meta-analysis can often provide more data to increase power and reduce false negative findings. This may indeed be the case in SCDN, as due to its clinical variability and disease etiology, much larger sample sizes may be needed to detect "true" associations and identify novel genetic association with disease. As a part of the SickleGen consortium, we were able to obtain summary level data from multiple SCD patient cohorts from across the United States. All study sites have patterned clinical data collection after case report forms originally developed for the Cooperative Study of Sickle Cell Disease (CSSCD), and so phenotypic harmonization was possible. Three previously described SCD cohorts were utilized in this analysis: Outcome Modifying Genes in Sickle Cell Disease (OMG-SCD),[16] Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy (Walk-PHaSST)[212] and Pulmonary Hypertension and the Hypoxic Response in Sickle Cell Disease (PUSH)[213]. Patients less than 16 years old were excluded from PUSH. GFR was estimated using the MDRD study definition and dichotomized at the clinically relevant threshold of 90 ml/min/1.73m^2 and proteinuria was identified by dipstick analysis. In total, 1064 patients

with complete data were included in the analysis (Table 9). A total of 479,389 SNPs in common were tested across all three cohorts.

Table 9: Patients with complete data included in SCDN GWAS meta-analysis

	OMG-SCD	Walk-PHaSST	PUSH
Number of subjects	463	496	105
Mean age (SD)	34.2 (12.0)	37.7 (12.6)	18.0 (1.4)
% Female	54.6	54.2	50.5
% Hb SS	85.0	71.5	81.0
Mean GFR (SD)	152.1 (67.3)	148.6 (63.2)	234.1 (79.9)
% GFR < 90 ml/min/1.73m^2	17.5	17.7	0

When we modeled GFR as a continuous variable across the meta-analysis including all three cohorts, the SNP with the most evidence for association was rs7553158 in the *TNNI3K-FPGT* locus ($p= 7.1x10^{-7}$). Fucose-1-phosphate guanylyltransferase (*FPGT*) converts GTP and β-l-fucose-1-phosphate to GDP-l-fucose, a process essential in the kidney to reutilize L-fucose from the turnover of glycolipids. It is also highly expressed in the porcine and human kidney.[214] Thus, this association represents an excellent biological candidate for kidney function. When we dichotomized GFR based on the definition of CKD stage 2, the most significant association within a gene was rs7201659 in *XYLT1* ($p=1.7x10^{-5}$). This results recapitulates our finding in the Duke single GWAS (OMG-SCD) and together with our results from the zebrafish modeling, shows strong evidence for its role in SCDN pathology.

Outside of the use of meta-analysis in GWAS has been the observation that many genetic loci appear to contain variants that are associated with multiple traits within patient populations. These associations have been identified in many disease areas, including rheumatoid arthritis,[215] Chron's disease,[216] systemic lupus erythematosus,[217] and type 1 diabetes.[218] The associations identified in these studies suggest that these disease traits, or traits within diseases, share common genetic pathways and highlight the relevance of pleiotropy in complex human disease. Given the characterization of widespread pleiotropy in complex traits and disease states, many statistical models have been developed to perform GWAS on multiple phenotypes within a single set of genetic data.[219-222] One of the most widely used methods, MultiPhen, provides a rapid and powerful approach by identifying the linear combination of the phenotypes most associated with genotype at each SNP.[223] Indeed, it has been shown to have a higher power than single-phenotype approaches in many scenarious.[223] It short, it reverses the regression, such that genotype is regressed on phenotype, allowing multiple phenotypes to be jointly modeled as predictors of the SNP genotypes.[223] As nephropathy outcomes in SCD are often correlated, we sought to perform a multivariate GWAS using the Duke dataset described previously (Table 5). Using GFR (dichotomized at CKD stage 2) and proteinuria as outcomes (r^2=0.48), we applied the MultiPhen model using imputed data generated from the Illumina-610 BeadChip. Notably, one of the most associated SNPs

within a gene was in *CRYL1*, which we found to be associated previously in our single-outcome GWAS (Table 10).

Table 10: Nominally significant *CRYL1* SNPs identified in multivariate SCD renal GWAS

Gene	SNP	P-value	Location
CRYL1	rs9315599	2.77×10^{-5}	intronic
CRYL1	rs9509235	2.77×10^{-5}	intronic

In summary, using meta-analysis and multivariate GWAS, we provide further genetic evidence for the role of several novel candidates in SCDN. Although still preliminary, these genes and markers may indicate novel genetic mechanisms contributing to SCD nephropathy, and may further diagnostic paradigms for identifying those patients most at risk.

5. Discussion and Future Directions for Sickle Cell Disease Nephropathy Research

5.1 Summary of contributions to sickle cell disease nephropathy genetic research

Sickle cell disease nephropathy (SCDN) remains one of the most severe clinical outcomes among SCD patients. However, despite its occurrence and increase in incidence over the last few decades, few studies have been conducted to determine potent risk factors that predispose patients to SCDN. The onset of CKD among SCD patients represents a complex etiology, although it is clear now that genetic factors play a part in the development of disease.[96, 224, 225] The ultimate goal of this dissertation was to identify putative genetic candidates for SCDN and gain a better understanding of factors

involved in disease heterogeneity and progression. To accomplish this, we have used a tiered approach employing genetic association strategy in patient populations and functional examination in relevant zebrafish models. Specific contributions are summarized below.

5.1.1 The role of *MYH9* and *APOL1* in sickle cell disease nephropathy

In this study, we provided the first *in vivo* evidence of *APOL1* involvement in human nondiabetic nephropathies. Specifically, we demonstrated the essential role of both *apol1* and *myh9* in the development of the pronephric glomerulus and proper renal filtration in zebrafish. Furthermore, we showed the first *in vivo* evidence of a possible adverse effect of the *APOL1* G2 allele. Importantly, and in relation to sickle cell disease, we identified a robust interaction between the two genes (*apol1* and *myh9*) that only became apparent under conditions of anemic stress. This is in concurrence with our previous study in SCD patents.[96] Finally, we showed an interaction effect of the *APOL1* G2 allele on *myh9* expression that was exacerbated under anemic stress, providing further support for a role of both of these genes in SCDN. This study provided essential biological insight into the mechanisms by which *MYH9* and *APOL1* confer disease risk and highlights the importance of comprehensive evaluation of functional consequences at a susceptibility locus.

5.1.2 Identification of putative genetic risk factors for SCDN

Using an array of unbiased genome-wide approaches, we found associations of multiple loci with nephropathy outcomes in a clinically well-characterized cohort of SCD patients. Although our main approach only focused on associations with two outcomes in SCD individuals (proteinuria and GFR), they represent robust clinical measurements and have been shown to be highly correlated with kidney decline, both in the context of SCD and in other nephropathies.[16, 226, 227] Indeed, in SCD populations, proteinuria is often the earliest marker of kidney dysfunction, whereas (due to initial hyperfiltration, see Chapter 1) GFR decline is observed in later stages of CKD progression.[16]

Upon interrogating both common (Illumina BeadChip) and rare (ExomeChip) genome-wide variation associated with proteinuria and GFR in SCD patients, we identified seven novel candidates nominally associated with SCDN (Table 6). These candidates were *CRYL1*, *XYLT1*, *LRRC6*, *FAM114A2*, *ACSF3*, *SLC2A9*, and *KERA* and are discussed more extensively in Chapter 4. Although the ExomeChip does cover rare variation compared to traditional GWAS arrays, it nonetheless contains pre-selected variants that may not represent the genomic architecture in all populations, especially in an African-American cohort such as SCD. Therefore, using this same cohort of SCD patients, we next performed deep sequencing targeting the coding regions of each of these candidate genes to capture functional rare variation associated with SCDN.

Importantly, all of our genetic candidates associated with rare variation identified by the ExomeChip were significant using the gene-based SKAT-O test after deep sequencing. Finally, we used our zebrafish model system to assign functional and biological relevance to each of these genetic candidates. By assessing the effect of gene perturbation on glomerular filtration barrier (GFB) integrity in zebrafish, we were able to confirm biological function to 4/7 candidates identified through our association studies. The reason for the lack of biological confirmation in 3/7 candidates is unclear, although likely represents the genetic divergence between zebrafish and humans. Indeed, in the case of *SLC2A9*, were not able to efficiently knockdown gene function in our zebrafish model due to loci duplication in the zebrafish genome. The lack of an apparent phenotype upon *kera* knockdown and the inability to rescue the phenotype caused by *fam114a2* perturbation also point toward inconsistency in gene function across species. It may be, however, especially in the case of *kera* and *fam114a2* that these genes to not directly impact kidney function in CKD patients and instead provide risk to nephropathy by indirect pathways.

Finally, our preliminary results using both meta-analysis (GWAS) and multivariate association studies give further affirmation of *XYLT1* and *CRYL1* associations with SCDN. Intriguingly, these candidates seem to be only associated with common variation within SCD patients, suggesting that regulatory elements within (or nearby) these loci may be responsible for disease risk. However, further studies will be

needed to determine if this is the case. In summary, we have identified a set of putative novel candidates associated with clinical outcomes in SCDN and provide functional evidence to a subset of these candidates using a relevant model system. These results may help us further identify SCD patients most at risk for developing nephropathy and give us insights into the mechanism by which SCDN may develop.

5.2 Future directions for sickle cell disease nephropathy research

5.2.1 Perspectives and insights into the mechanism of *APOL1*-associated nephropathy

A focal point of many studies in the last few years has been to address the mechanism by which *APOL1* may induce nephropathy in African Americans. Through many reports in human patients it is clear now that the *APOL1* variants, G1 and G2, confer a large proportion of CKD risk in African American populations and many groups have been attempting to address the role of these variants in CKD. Indeed, we were the first to show that *in vivo*, the *APOL1* G2 allele may have a dominant-negative (or at least toxic) effect on glomerular function in our zebrafish model. Subsequently, many other groups have come to this same conclusion. Using podocyte cell culture models, Lan *et al.* showed that both *APOL1* risk variants induced podocyte necrosis when overexpressed *in vitro*.[138] Similarly, Olabisi and colleagues reported a cytotoxic effect of the *APOL1* risk alleles in human embryonic kidney cells.[228] The zebrafish system has also been utilized by other groups to assess the effect of the *APOL1* variants on

pronephric structure and function. Kotb *et al.* recapitulated much of our data by knocking down *apol1* in zebrafish larvae, showing severe edema and dysfunction of the glomerular filtration barrier. However, they showed a rescue effect by co-expressing human nephrin (*NPHS1*) in *apol1* morphants, suggesting that *APOL1* may effect nephrin expression to induce nephropathy.[229] Another study utilized transgenic zebrafish specifically expressing the human *APOL1* variants in podocytes and endothelial cells and report segmental foot process effacement and other glomerular irregularities.[230] Finally, using transgenic mice in which *APOL1* G1/G2 were expressed under the nephrin promoter, Bruggeman and colleagues observed severe podocyte loss in aged *APOL1*-G2 mice compared to those expressing the *APOL1* wild-type variant.[231] Concurrent with our findings, all of these studies point toward an adverse effect of *APOL1* G1/G2 expression on kidney function and kidney cell types.

Despite multiple studies showing cytotoxicity and kidney abnormalities upon *APOL1* G1/G2 expression, we still do not have a clear idea of the mechanism by which this occurs. In podocyte cell culture studies, researchers noted that *APOL1* G1/G2 induced cathepsin L diffusion, suggesting that these variants caused enhanced lysosomal membrane permeability.[138] Indeed, this hypothesis would align with the role of *APOL1* in trypanolysis (see Chapter 2). Evidence also points to the activation of stress-activated protein kinases, p38 MAPK, and JNK by potassium efflux induced by *APOL1*

G1/G2 expression in embryonic kidney cells.[228]. Together, these studies have begun to identify possible pathways by which *APOL1* may mediate nephrotoxicity.

Our finding that *APOL1* may interact with other genetic partners, especially in the presence of anemia is quite intriguing. Indeed, it has been postulated by our group and many others that in human patients, *APOL1* risk variants may induce disease only under an external stress (e.g. HIV infection, anemia/hemolysis in SCD, etc.)[96, 228, 230] Given these hypotheses, we have begun interrogating transcriptional profiles under *APOL1* modulation. Although our initial studies in CRISPR/Cas9 zebrafish mutants were done in the F0 generation, we now have developed F2 *apol1* homozygous mutants. Importantly, these fish were generated on the pod::NTR-mCherry/l-fabp::VDBP-GFP background and recapitulate our studies in the F0 population, displaying proteinuria as a result of compromised GFB integrity (Figure 22). Using this newly developed transgenic line, we will be able to compare transcriptional profiles in *apol1* mutant and

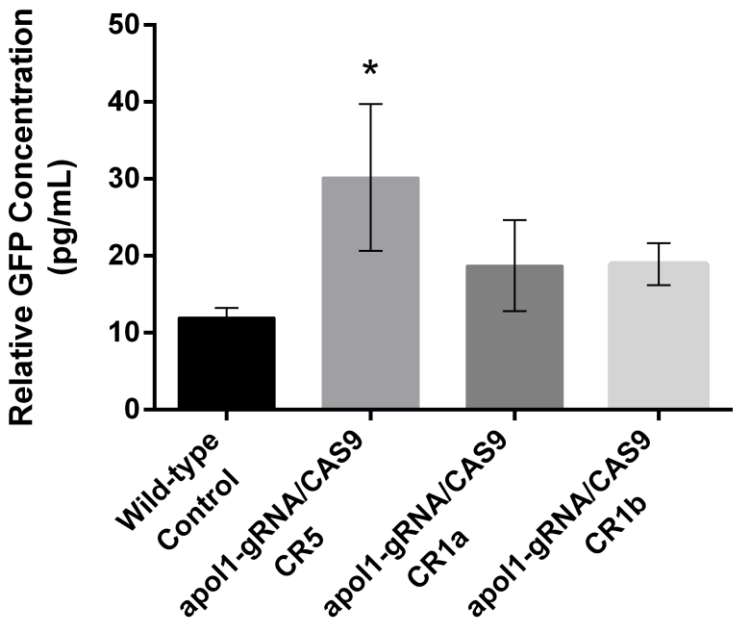

Figure 22: Assessment of glomerular filtration barrier integrity in *apol1* F2 mutant embryos

apol1 F2 mutants were generated on the pod::NTR-mCherry/l-fabp::VDBP-GFP background. Three separate F2 generations were generated, each displaying different homozygous deletions in exon 3 of *apol1* (CR5, CR1a and CR1b). At 3 days-post-fertilization (dpf), 20 embryos per condition (in replicate) were place in separate wells. After 24 hours, 100µl of embryo medium was assayed with a GFP ELISA kit and relative GFP concentration was quantified, which corresponds to the amount of VDBP (albumin) present. Experiment was repeated two times. *$p<0.05$

apol1 mutant/anemic larvae to assess expression changes caused by *apol1* knockout specifically in the context of anemia. In addition, we have generated fluorescence-activated cell sorting (FACS) profiles for the pod-mCherry cassette present in these embryos, allowing us to isolate and extract RNA specific to podocytes (Figure 23). We will then perform RNA-Seq to identify podocyte-specific transcriptional changes *in vivo*

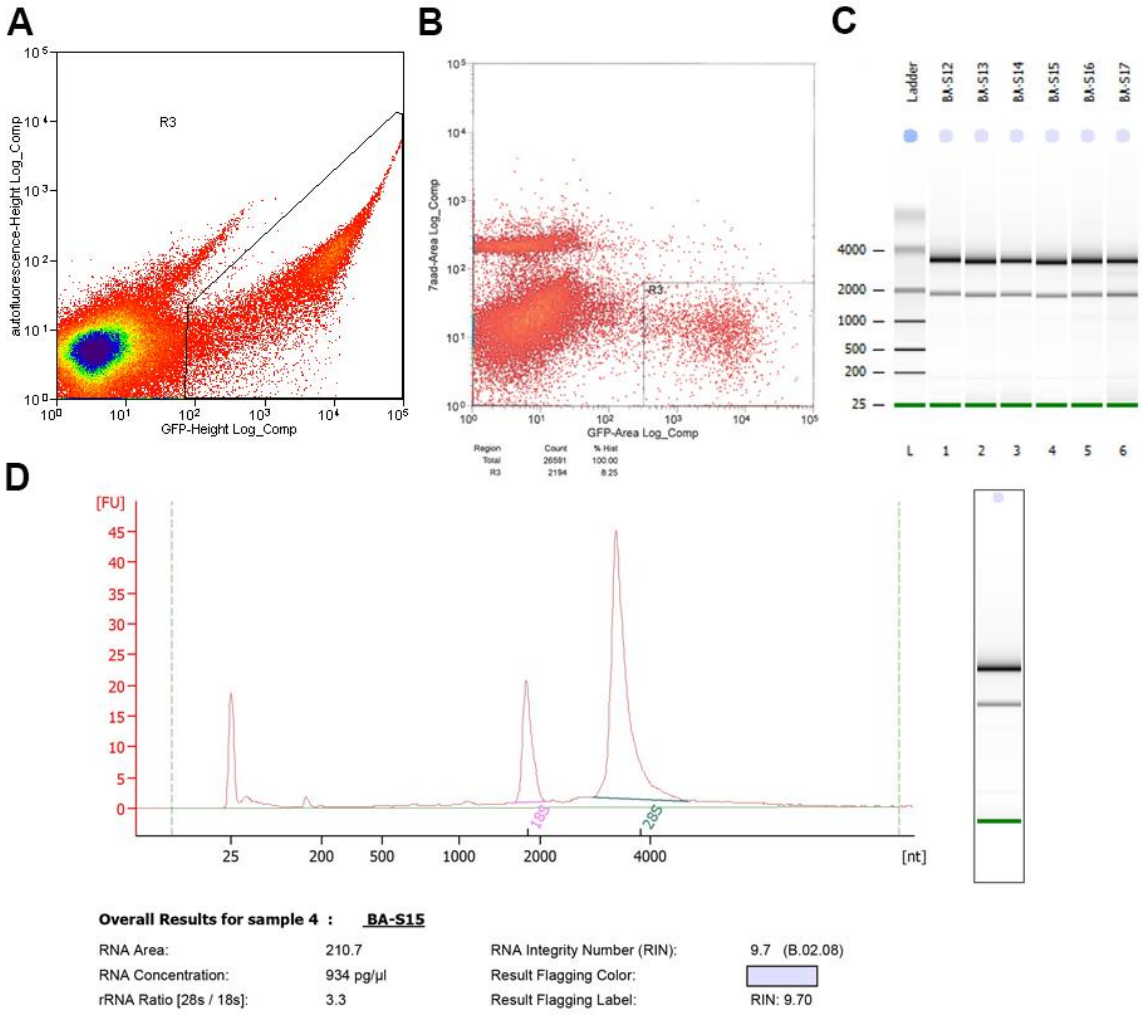

Figure 23: FACS and RNA quality profile of *apol1* CRISPR/Cas9 zebrafish embryos

60-70, 4 dpf pod-mCherry transgenic embryos were physically dissociated in 0.25% trypsin/EDTA and cell suspensions were filtered through a 30µm filter before sorting. mCherry-positive cells were gated based on (A) log_comp values separated from auto-fluorescense as well as (B) cell death labeled by 7-aminoactinomycin D. Approximately 10-20k mCherry-positive cells were successfully sorted from each sample. (C-D) TapeStation electrophoresis and electropherogram analysis show exceptional RNA integrity (RIN 9.10-9.70) after RNA isolation and extraction from each sample, with a total RNA yield of 7-12 ng.

resulting from *apol1* depletion which may point toward novel pathways and/or

interaction partners with *APOL1*.

Alongside studies in *apol1* CRISPR/Cas9 zebrafish, we have also begun interrogating transcriptional changes as a result of the nephrotoxic effect of the *APOL1* G2 allele. Using a similar approach as described above, we injected either human *APOL1* G2 or *APOL1* G0 (wild-type) mRNA into both pod-mCherry and Fli-eGFP embryos. At 4 days-post-fertilization, we used FACS to isolate podocytes and endothelial cells respectively from these embryos and extracted RNA. Again, by performing RNA-Seq on these cell types, we hope to gain an understanding of transcriptional changes resulting from *APOL1* G2 expression.

Finally, we have also begun to look for interaction partners with *APOL1* in the Duke-OMG SCD patient dataset. We performed an interaction GWAS using the *APOL1* G2 allele as a covariate and GFR as the outcome. This joint test for association allowed for an interaction between *APOL1* G2 and any other genome-wide SNP to predict GFR among SCD patients. Interestingly, we found several associated loci across the genome that significantly predicted GFR when interacting with *APOL1* G2 (Figure 24). The most significant SNP (rs10790180; $p=1.23x10^{-7}$) within a gene resided in centrosomal protein, 164 (*CEP164*). This is especially intriguing, as *CEP164* has been associated with nephronophthisis and when knocked down in zebrafish larvae, causes nephric cyst formation.[232, 233] In order to functionally validate this finding, we have begun assessing the effect of *APOL1* G2 expression in the presence of *cep164* perturbation in our zebrafish model.

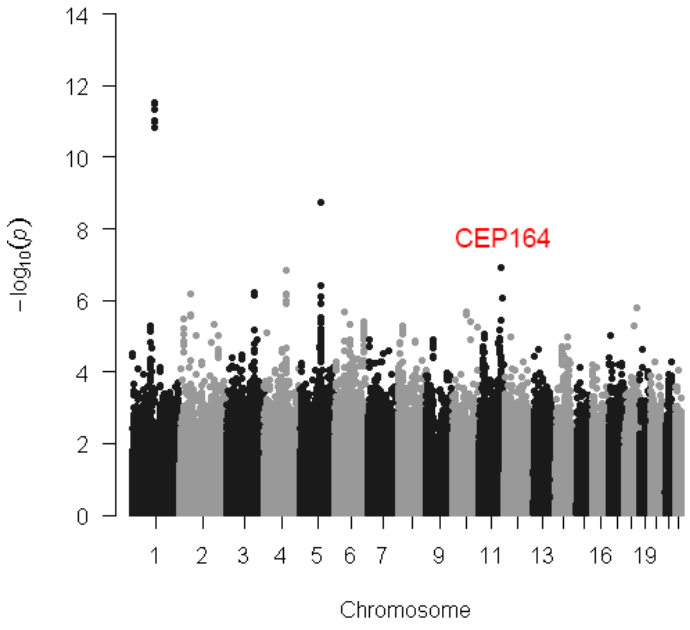

Figure 24: Manhattan plot of GFR gene-gene interaction GWAS with *APOL1* G2 in SCD patients

A joint test for association was performed in the Duke-OMG cohort of SCD patients. The allowed for an interaction between *APOL1* G2 and any other genome-wide SNP to predict GFR among SCD patients. The most significant SNP (rs10790180; p=1.23x10-7) within a gene resided in centrosomal protein, 164 (CEP164).

Defining the mechanism by which *APOL1* induces nephropathy in African Americans has been an intensely studied area of research over the last few years and will continue to be into the near future. We have begun several preliminary studies attempting to identify transcriptional changes associated with either *apol1* knockout or *APOL1* G2 expression, which may give us insight into dysregulated genes and/or

pathways causal to kidney decline. Further understanding of *APOL1*-induced nephropathy is crucial both for SCDN pathology and other forms of CKD.

5.2.2 Further characterization of pleiotropy in SCDN

Sickle cell disease nephropathy represents a complex etiology as discussed in Chapters 1 and 2. Although our previous efforts have tried to identify risk factors for distinct renal outcomes, it is becoming apparent in many other complex disease that cross-phenotype and/or pleiotropy effects are significant and may result in more power to detect association. A long-standing observation among SCD is the presence of pulmonary hypertension (PH) in about 30% of the patient population.[16, 173, 234] Intriguingly, the development of PH is often correlated with CKD incidence in patient populations.[235] Indeed, in the Duke-OMG dataset, we find a high correlation (r^2=0.48) between abnormal echocardiogram results and proteinuria in SCD patients. Thus, it may be beneficial to incorporate multiple correlated clinical outcomes in the same analysis in order to elucidate strong predictors of risk for SCDN. However, clinically well-characterized cohorts of patients are needed in order to directly compare outcomes, and so communication between collaborating SickleGen centers is of paramount importance as the field moves forward.

5.2.3 The need for novel treatments for sickle cell disease nephropathy

As discussed in Chapter 1, sickle cell disease nephropathy patients are plagued by an unfortunate paradox, in that traditional treatments for SCD complications

(hydroxyurea, transfusions, etc.) do not improve renal outcome and that traditional treatments for chronic renal disease (ACE-inhibitors) are seemingly ineffective in SCD patients.[64, 236-241] Thus, the need for novel treatments to combat progressive renal failure in SCD patients is great and should be the focus of research studies in the future.

To accelerate the potential for translational research, we have begun studies to screen the Prestwick Library of 1,200 commercially available FDA-approved small molecules to identify compounds that can ameliorate the nephropathy phenotypes of our CRISPR/Cas9 zebrafish model of *apol1* loss-of function. In addition, we have begun developing stable *APOL1* G2 transgenic zebrafish models for use in this screen as well. Although these studies primarily focus on amelioration of *APOL1*-induced nephropathy, we plan to extend this model to other novel targets (see Chapter 4) to identify novel compounds that may reduce renal decline in SCD. This zebrafish system allows rapid screening of chemical compounds and is a powerful platform to investigate therapeutic targets for newly identified genetic risk factors.

5.2.4 General implications for SCDN risk factors and treatment

Possibly the most pressing need among SCD patients progressing toward renal failure is earlier identification of disease markers. As we begin to address the genetic complexities of SCDN and gain more understanding of the mechanisms responsible for disease, this information could be amalgamated into genetic tests to provide faster and more accurate diagnosis. Furthermore, a better understanding of the mechanisms

involved could lead to treatment options that could be more specific to SCDN, compared to other nephropathies. Ultimately, this research could reduce the amount of patient suffering due to chronic renal failure in SCD.

Appendix A: Chapter 3 Materials and Methods

A.1 Zebrafish Stocks

We maintained wild-type (WT) zebrafish stocks (Ekkwill, Ekkwill x AB F1 outcross, or pod::NTRmCherry[144]) according to standard zebrafish husbandry procedures. Embryos were obtained from natural matings of adult fish.

A.2 Morpholino oligonucleotide-mediated knockdown and human mRNA complementation

Complementation assays were designed essentially as described.[242] Briefly, a MO was designed by Gene Tools, LLC (Philomath, OR) to target the translation initiation site of zebrafish *apol1* (*apol1*-MO), (5′-AGTCGTCCAGCCATTCCATGAGGGT-3′). A translation-blocking morpholino (MO) targeting zebrafish *myh9* and a splice-blocking MO targeting zebrafish *atpif1a* were described previously.[123, 159] *APOL1* G1 and G2 allelic constructs were synthesized from a WT *APOL1* human ORF clone (GenBank: BC112943) using site-directed mutagenesis (Stratagene, QuikChange II), subsequently transcribed (mMESSAGE mMACHINE®, Life Technologies, Ambion®) into capped mRNA and co-injected with *apol1*-MO into zebrafish embryos at the one-to-four cell stage (WT, 100pg/μl; G1, 100pg/μl; G2, 100pg/μl). Controls were injected with phenol red. A WPI pneumatic pico pump microinjector was used for MO and mRNA injection to deliver 1 nl/embryo. After injection, embryos were maintained at 28°C in embryo medium.

A.3 Dextran microinjection and time-lapse filtration scoring

48 h.p.f. larvae were anesthetized in 1.0% tricaine and placed laterally in agarose wells. 70 kDa FITC-conjugated dextran (LifeTechnologies, 3.0nl/embryo) was injected into the cardiac venous sinus and larvae were transferred to embryo medium for recovery after injection. The eye vasculature of individual fish was imaged at two, 12, 24, 36, and 48 hours after dextran injection using a Nikon AZ100 fluorescent microscope and Nikon NIS Elements AR software. The average fluorescence intensity was measured across the eye (ImageJ) and changes in intensity relative to the two h.p.i measurements were calculated for comparison. GraphPad Prism version 6.03 (GraphPad Software, San Diego, CA) was used for statistical analysis of relative intensity.

A.4 Fluorescence-activated cell sorting (FACS)

Glomeruli from *pod*::NTR-mCherry adult zebrafish were manually dissected and dissociated in 0.5% trypsin/collagenase. Dissociated cells were then filtered through a 70μm strainer and filtered again through a 30μm strainer. Cell-sorting was done on a Beckman Coulter Astrios instrument for mCherry (610nm). Sorted cells were placed in RLT Buffer (Qiagen) and RNA was extracted using the RNeasy Micro Kit (Qiagen).

A.5 Reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA from zebrafish embryos was extracted with TRIzol® Reagent (Life Technologies) and cDNA was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen). The following primers were used for amplification: *actb1*,

Fwd: TTGTTGGACGACCCAGACAT, Rev: TGAGGGTCAGGATACCTCTCTT; *nphs2*, Fwd: CCTTCGCTAGCATTCCAGAC, Rev: GCAGCTCTGGAGGAAGATTG; *wdr81*, Fwd: ATGGAGAGAAAAACATGGAGGA, Rev: AAGGAGAAAACCTGGAAGAACC; *apol1*, Fwd: GACTTTCGATTAAGTGAAACTCAGAGAGA, Rev: GTTATGGTAGCTACACCTCCCACAGCGCTG; *myh9* (qRT), Fwd: GGAAAAACCGAAAACACCAA, Rev: CAATATTGGCTCCAACGATGT. qRT-PCR was performed on a ABI Prism 7900HT instrument and cycle threshold values were computed using SDS 2.3 software (Applied Biosystems). *myh9* relative expression was calculated against *actb1* in each sample and compared against sham-injected controls to determine significant differences in expression.

A.6 Transmission electron microscopy of glomerular ultrastructure

5 dpf embryos were anesthetized in 1.0% tricaine and then fixed in 4.0% glutaraldehyde in 0.1M Na_2PO_4 buffer containing 0.12mM $CaCl_2$ at 4°C overnight. Fixed larvae were washed in 1X PBS, washed in 1X phosphate buffer, postfixed in 2% osmium tetroxide for 2 hours, and dehydrated through a graded acetone series. Embedding was performed with Epoxy 812. Sections were cut on a Leica-Reichert Ultracut E ultramicrotome and semithin sections (1.0μm) were collected and stained with toluidine blue. 90nm ultrathin sections were placed on copper grids and contrasted with 4.0% uranyl acetate for 10 minutes. Grids were incubated in lead citrate (Reynolds Lead) for 3

minutes and then examined on a Phillips CM12 electron microscope. Images were taken with an AMT XR61 camera.

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Biography

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Publications:

Genetic Dissection of the Renal Disease of DiGeorge Syndrome. New England Journal of Medicine. Lopez-Rivera, E.*, Yangfan, L.P.*, Anderson, B.R.* et al. 2016. [In revision]

In vivo Modeling Implicates APOL1 *in Nephropathy: Evidence for Dominant Negative Effects and Epistasis under Anemic Stress.* PLOS Genetics. Anderson, B. R., Howell, D.N., Soldano, K., Garrett, M.E., Katsanis, N., Telen, M.J., Davis, E.E., Ashley-Koch, A.E.

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Honors and Awards:

Bass Instructional Fellowship, Duke University Graduate School, Spring 2015
Travel Grant for Conference Presentation, awarded by Duke University Program in Genetics and Genomics, Fall 2013; Fall 2014
Travel Award for NIGMS Short Course on Statistical Genetics and Genomics, University of Alabama-Birmingham, 2013