In vivo Modeling Implicates APOL1 in Nephropathy: Evidence for Dominant Negative Effects and Epistasis under Anemic Stress

Blair R. Anderson¹, David N. Howell², Karen Soldano¹, Melanie E. Garrett¹, Nicholas Katsanis¹, Marilyn J. Telen³, Erica E. Davis¹‡, Allison E. Ashley-Koch¹‡*

¹Center for Human Disease Modeling, Duke University Medical Center, Durham, North Carolina, United States of America, ²Department of Pathology, Division of Pathology Clinical Services, Duke University, Durham, North Carolina, United States of America, ³Department of Medicine, Division of Hematology, Duke University Medical Center, Durham, North Carolina, United States of America

‡ These authors are joint senior authors on this work.
* allison.ashleykoch@duke.edu

Abstract

African Americans have a disproportionate risk for developing nephropathy. This disparity has been attributed to coding variants (G1 and G2) in apolipoprotein L1 (APOL1); however, there is little functional evidence supporting the role of this protein in renal function. Here, we combined genetics and in vivo modeling to examine the role of apol1 in glomerular development and pronephric filtration and to test the pathogenic potential of APOL1 G1 and G2. Translational suppression or CRISPR/Cas9 genome editing of apol1 in zebrafish embryos results in podocyte loss and glomerular filtration defects. Complementation of apol1 morphants with wild-type human APOL1 mRNA rescues these defects. However, the APOL1 G1 risk allele does not ameliorate defects caused by apol1 suppression and the pathogenicity is conferred by the cis effect of both individual variants of the G1 risk haplotype (I384M/S342G). In vivo complementation studies of the G2 risk allele also indicate that the variant is deleterious to protein function. Moreover, APOL1 G2, but not G1, expression alone promotes developmental kidney defects, suggesting a possible dominant-negative effect of the altered protein. In sickle cell disease (SCD) patients, we reported previously a genetic interaction between APOL1 and MYH9. Testing this interaction in vivo by co-suppressing both transcripts yielded no additive effects. However, upon genetic or chemical induction of anemia, we observed a significantly exacerbated nephropathy phenotype. Furthermore, concordant with the genetic interaction observed in SCD patients, APOL1 G2 reduces myh9 expression in vivo, suggesting a possible interaction between the altered APOL1 and myh9. Our data indicate a critical role for APOL1 in renal function that is compromised by nephropathy-risk encoding variants. Moreover, our interaction studies indicate that the MYH9 locus is also relevant to the phenotype in a stressed microenvironment and suggest that consideration of the context-dependent functions of both proteins will be required to develop therapeutic paradigms.
Author Summary

African Americans have a disproportionate risk for developing chronic kidney disease compared to European Americans. Previous studies have identified a region on chromosome 22 containing two genes, MYH9 and APOL1, which likely accounts for nearly all of this difference. Previous reports provided strong statistical evidence implicating APOL1 as the major contributor to nephropathy risk in African Americans, driven by two coding variants, termed G1 and G2. However, other groups still report statistical evidence for MYH9 association in kidney disease, and animal models have demonstrated biological relevance for MYH9 function in the kidney. Here, we show that suppressing apol1 in zebrafish embryos results in perturbed kidney function. Importantly, using this in vivo assay, we show that the G1 variant appears to cause a loss of APOL1 function, while the G2 variant results in an altered protein that may be acting antagonistically in the presence of normal APOL1. We also report a genetic interaction between apol1 and myh9 under anemic stress, which is consistent with our previous findings in sickle cell disease (SCD) nephropathy patients. Finally, we provide functional evidence in vivo that the G2-altered APOL1 may be interacting with MYH9 to confer nephropathy risk.

Introduction

Chronic kidney disease (CKD) is an acute public health problem world-wide. Within the United States alone, it affects up to 14% of the adult population and is associated with both high costs and poor clinical outcomes[1]. Compared with European Americans, African Americans have a disproportionate risk for several forms of CKD, including human immunodeficiency virus (HIV)-associated nephropathy, focal segmental glomerulosclerosis (FSGS), hypertension-attributed CKD, and sickle cell disease nephropathy (SCDN), all of which contribute to a four-fold increased risk of the most severe stage of CKD, end-stage renal disease (ESRD)[1–5]. A genomic region on chromosome 22q12 likely accounts for almost all of this racial disparity. This region contains two genes, non-muscle myosin heavy chain IIA (MYH9; Entrez, 4627) and apolipoprotein L1 (APOL1; Entrez, 8542), both of which have been associated with increased risk among African American patients with nondiabetic nephropathy[5–11]. Initial admixture mapping and subsequent fine mapping studies focused on MYH9[8, 9, 11]. Initial admixture mapping and subsequent fine mapping studies focused on MYH9[8, 9, 11]. However, due to the inability to identify variants in MYH9 that alter protein sequence, the major source of genetic association has been attributed to APOL1, located 14 kb downstream of MYH9[6]. Two APOL1 alleles, G1 (encoding p.S342G and p.I384M in cis) and G2 (encoding p.N388del:Y389del), comprise one of the strongest genetic signals ever reported in complex human disease (odds ratios ranging from 10.5 to 16.9)[6, 7]. Additionally, these alleles alter the protein to confer resistance to Trypanosoma brucei rhodesiense, offering a potential evolutionary explanation for the increased occurrence observed among individuals of African ancestry [6].

Despite these genetic findings and the association of this locus with increased risk of multiple forms of CKD, 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[12], and heterozygotes appear viable without any detected abnormalities [13]. However, subsequent studies have demonstrated that knock-in mutants display renal glomerulosclerosis, while podocyte-specific deletion of Myh9 may predispose mice to glomerulopathy[14–16]. 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[17]. 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[18, 19], while overexpression of APOL1 and its risk alleles enhance podocyte necrosis in vitro [20].

Nephropathy is a major contributor to early mortality in patients with sickle cell disease (SCD)[21, 22]. SCDN is a clinically well-characterized pathology that includes glomerular hypertrophy, hyposcstenuria, tubular dysfunction, proteinuria, and overall progressive renal failure[23]. We reported previously an association of both MYH9 and APOL1 variants as independent risk factors for proteinuria in a SCD study population[5]. Additionally, when glomerular filtration rate (GFR) in SCD patients was modeled as a function of the previously reported MYH9 risk haplotype and the APOL1 recessive model, we observed a significant interaction between the two genes, suggesting that APOL1 and MYH9 may act together to induce SCDN [5]. However, as with other forms of CKD, well-characterized in vivo model systems are needed to understand both the individual effects of APOL1 relevant to disease, and also the potential interaction of APOL1 with MYH9 in the context of anemic stress as observed in SCD.

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 (RefSeq: NM_001030138) and myh9 (RefSeq: NM_001098177.2)[24, 25]. 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 non-diabetic nephropathy and intimate context-dependent roles in disease pathology.

**Results**

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 pri-mates[26, 27]. 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 (Fig 1A–1D). 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 expression at time points corresponding to the formation of the pronephros. Additionally, we detected apol1 expression in flow-sorted podocyte fractions harvested from glomeruli of pod::NTR-mCherry adult zebrafish (Fig 1E) [28].

To test the effects of apol1 suppression, we designed a translation-blocking morpholino (MO; Gene Tools, LLC) 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[24, 30] (Fig 2A–2C). 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; Fig 2D), 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. Importantly, 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 (S1 Fig). 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 (S2 Fig). 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[31, 32] (Fig 3A–3C). 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; Fig 3D).
In Vivo Modeling of APOL1 in Nephropathy

A
Control

B
apol1-MO

C

Percentage of Embryos with Edema

D

Percentage of Embryos with Edema

E

Relative Fluorescent Intensity

F

Graph showing the effect of APOL1 mRNA and apol1-MO on the percentage of embryos with edema.

G
Control

H
apol1-MO

I
apol1-MO + WT mRNA
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 [30]. 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; Fig 2E and 2F). 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; Fig 2E and 2F), consistent with the occurrence of proteinuria. Importantly, this phenotype was also reproduced in apol1 CRISPR/Cas9 larvae (Fig 3E). 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; Fig 2E and 2F).

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 [17], 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 (S3 and S4 Figs). 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 (Figs 2G, 2H, and S4). However, we did not observe GBM thickening as evident in myh9-MO injected larvae (S3 Fig). In areas in which we did observe foot process formation, podocyte protrusions were irregular and inhibited slit diaphragm development (Figs 2G, 2H, and S4).

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...
Fig 3. apol1-CRISPR F0 zebrafish embryos reproduce phenotypes observed in apol1 morphants. (A) 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.

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last exon of the gene[5–7, 10]. 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; Fig 1D). 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/ml). Importantly, each APOL1 allelic construct produces a stable protein detectable by immunoblotting when co-injected with apol1-MO (S2 Fig). 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 (Fig 4A and 4B). 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 (Fig 4A and 4D) 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; Fig 4B); no edema was observed with injection of 100pg APOL1 G1 mRNA alone (Fig 4A). 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; Fig 4E and 4F). 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 (Fig 4G) 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 (S5 Fig). In concurrence with our observations of gross morphological defects, embryos injected with G2 mRNA alone also
Fig 4. 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. (H) Zebrafish embryos injected with APOL1 G2 mRNA (100pg/ml) 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. (I) Embryos injected with APOL1 G1 mRNA (100pg/ml) alone display normal glomerular ultrastructure. Scale bar, 500nm. White bars, normal; black bars, edema. C, sham-injected control; NI, non-injected control. *p<0.05.

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display glomerular aberrations and microvillus protrusions (Fig 4H) similar to myh9 and apol1 morphants (Figs 2H and S4); no abnormalities were seen in larvae injected with G1 mRNA alone (Fig 4I). 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.

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

Although recent studies have provided statistical evidence implicating APOL1 variation in nondiabetic nephropathies[7, 33, 34], MYH9 risk variants are still associated with chronic kidney disease (CKD) in non-African American populations[35] and in sickle cell disease nephropathy[5]. 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[5]. Therefore, we tested for functional interaction effects between apol1 and myh9 in zebrafish, an experimentally tractable model for investigating additive and synergistic effects[36–40]. 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 (Fig 5A), even when individual MO concentrations were reduced to subeffective doses (Fig 5B). 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/ml) and apol1-MO was co-injected with human MYH9 WT mRNA (100pg/ml). However, we were unable to rescue the suppression phenotypes of either apol1 or myh9 with the human mRNA of the reciprocal gene (S6 Fig).

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[41, 42]. While a zebrafish model of SCD does not exist currently, suppression of ATPase inhibitory factor 1 (atpif1a), a mitochondrial protein, produces profound anemia in zebrafish by interfering with heme synthesis through decreased catalytic efficiency of ferrochelatase[43]. The resultant effect of low hemoglobin and hematocrit stresses the kidney because of the organ’s high oxygen consumption. Consistent with the original report[43], we observed a dose-dependent reduction in hemoglobin with increasing concentrations of the atpif1a MO (atpif1a-MO), as measured by o-dianisidine staining of whole MO-injected larvae at 4 dpf. Strikingly, we found
**Fig 5. 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.
lарvae 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 a significantly increased edema formation compared to each MO alone. We next tested for an interaction between apol1 and myh9 in the context of atpif1α 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); atpif1α MO injected (n = 14); apol1-MO+myh9-MO (n = 12); apol1-MO+myh9-MO+atpif1α-MO (n = 11); Butafenacil (n = 48); But +myh9-MO+apol1-MO (n = 18). hpf, hours post-fertilization; hpi, hours post-injection. *p<0.001.

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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/atpif1α MOs; Fig 5C and 5E). 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; Fig 5E). 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[44]. 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; Fig 5D and 5F).

**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 quantitative real-time 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 (Fig 6A) and 3 dpf (Fig 6B). 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; Fig 6B), 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; Fig 6B), and a significant reduction of myh9 expression compared to APOL1 G2 mRNA alone (p = 0.0297; Fig 6B), 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 (Fig 6A), 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 anhn[45], trpc6b[46], and wt1a [47] upon apol1/APOL1 modulation (S7 Fig), suggesting that APOL1 G2 regulation may be specific to myh9.
In Vivo Modeling of APOL1 in Nephropathy

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 of APOL1 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 (Fig 5C), 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[20].

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[5–10, 33, 35, 48–50]. Additionally, APOL1 has been associated with an increased burden of cardiovascular disease in African Americans participating in the Jackson Heart Study[51]. 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[14–17]. 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 [26, 27], 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 (Fig 1A).

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[6].

Strikingly, injection of human APOL1 G2 mRNA alone resulted in significant edema formation in 5dpf 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 (Fig 1B and 1D). 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[5].

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[26]. More recent studies, however, have characterized the cellular localization of APOL1 in human kidney sections to podocytes, proximal tubules, and arteriolar endothelial cells[18]. 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[52, 53]. 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[8, 9], and since the nearby nonsynonymous variants identified in APOL1 provided stronger statistical association[5–7], 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[6, 33]. However, recent reports still demonstrate statistical association of MYH9 in nondiabetic nephropathy[5, 35] and previous in vivo modeling studies provide further evidence for the role of Myh9 in glomerular development and glomerulosclerosis[14–17]. 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[5, 10]. 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[21, 22, 41, 54].
Furthermore, we provide evidence suggesting that the functional consequences of \textit{APOL1} variation may not be acting in a strictly recessive manner as had been previously suggested\cite{5-7, 55}. Our data demonstrate that \textit{APOL1} G1 (I384M/S342G) confers loss of proper \textit{APOL1} function in the developing zebrafish kidney, while \textit{APOL1} G2 is acting in a dominant-negative manner to induce nephropathy, possibly through suppression of \textit{myh9}. These data indicate that the risk conferred by the \textit{APOL1}/\textit{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 \textit{myh9} in the development of the pronephric glomerulus and proper renal filtration in zebrafish. We report comprehensive \textit{in vivo} causal evidence of \textit{apol1} involvement in kidney decline, and we provide the first \textit{in vivo} evidence of a potential dominant-negative effect of the \textit{APOL1} G2 allele. Further, we have shown that the presence of the G2 allele decreases significantly the expression of \textit{myh9}. Similar to the common haplotype on 10q26 that influences age-related macular degeneration underscored by complex regulatory events of neighboring genes \textit{ARMS2} and \textit{HTRA1}, our data highlight further the importance of comprehensive evaluation of functional consequences at a susceptibility locus\cite{56}. Taken together, these data provide essential biological insight into the mechanisms by which \textit{MYH9} and \textit{APOL1} confer disease risk and progression in human nondiabetic nephropathies.

**Materials and Methods**

**Zebrafish stocks**

We maintained WT zebrafish stocks (Ekkwill, Ekkwill x AB F1 outcross, or \textit{pod}:\textit{NTR-mCherry}\cite{28} according to standard zebrafish husbandry procedures. Embryos were obtained from natural matings of adult fish.

**Morpholino oligonucleotide-mediated knockdown and human mRNA complementation**

Complementation assays were designed essentially as described\cite{57}. Briefly, a MO was designed by Gene Tools, LLC (Philomath, OR) to target the translation initiation site of zebrafish \textit{apol1} (NM_001030138) (\textit{apol1}-MO), (\textit{5'}-AGTCGTCCAGCCATTCCATGAGGGT-3\textit{'}). A translation-blocking morpholino (MO) targeting zebrafish \textit{myh9} and a splice-blocking MO targeting zebrafish \textit{atpif1a} were described previously\cite{17, 43}. \textit{APOL1} G1 and G2 allelic constructs were synthesized from a WT \textit{APOL1} human ORF clone (GenBank: BC112943) using site-directed mutagenesis (Stratagene, QuikChange II), subsequently transcribed (m\textit{MESSAGE} \textit{mMACHINE}, Life Technologies, Ambion) into capped mRNA and co-injected with \textit{apol1}-MO into zebrafish embryos at the one-to-four cell stage (WT, 100pg/ nl; G1, 100pg/nl; G2, 100pg/nl). 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.

**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 24, 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
24 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.

**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).

**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: TTGTTGGACGACCCACACAT, Rev: TGAGGCTCACGATCCTCTCTT; *nphs2*, Fwd: CCTTCCGTAGCATCCAGAC, Rev: GCAGCTCTGGAGGAAGATTG; *wdr81*, Fwd: ATGGAGAGAAAAACATGGAAGA, Rev: AAGGAGAAACCTGGAGAAAAAC; *apol1*, Fwd: GACTTGCAGTCACTACCCACACAGGTG; Rev: GTTATGGTACGCTACCTCCCACAGGCTG; *myh9* (qRT), Fwd: GGAAA AACCCGAAAAACACAA, Rev: CAATAATTGGCTCCACAGATGT; *anln* (qRT), Fwd: TTTGACCTTCACCACCACATT, Rev: TTTGCTGTAGTCACCTTTGA; *wt1a* (qRT), Fwd: ATGGCACACTTGTCAGAAGAA, Rev: TATATTTCTGTGGTTCCTGTG; *trpc6b* (qRT), Fwd: GCACATGCAGAGCCGCCGCGTGGG, Rev: CTAGGTGGCGCCATTGG CACTTGAAGAA. qRT-PCR was performed on a ABI Prism 7900HT instrument and cycle threshold values were computed using SDS 2.3 software (Applied Biosystems). Relative expression was calculated against *actb1* in each sample and compared against sham-injected controls to determine significant differences in expression.

**Transmission electron microscopy of glomerular ultrastructure**

5 dpf embryos were anesthetized in 1.0% tricaine and then fixed in 4.0% gluteraldehyde in 0.1M Na2PO4 buffer containing 12mM CaCl2 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.

**Genome-editing of the apol1 locus using the CRISPR/CAS9 system**

*apol1* gRNA was produced by synthesizing and annealing two oligonucleotides, gRNA F: TAGGTTTGGCAAGCCAAACAGTTCC and gRNA R: AACACAGGACTTGGCTTGGC ATCA. The annealed oligos were then ligated to a T7cas9sgRNA2 vector by performing the ligation and digestion in a single step in a thermal cycler as described [31]. 2 μL of the reaction was used for transformation. Prior to transcription, the gRNA vector was linearized with *BamHI*. gRNA was transcribed using the MEGAshortscript T7 kit (Life Technologies, AM1354) and purified using alcohol precipitation. A total of 100pg of *apol1* gRNA and 200pg of CAS9 protein (PNA Bio) was co-injected into individual cells of one-cell stage embryos. For T7
endonuclease I assay, genomic DNA was prepared from 1 dpf embryos as described [58]. A short stretch of the genomic region (~270–280 bp) flanking the apoll gRNA target site was PCR amplified from the genomic DNA (Fwd: TGTGTGAAGGATGCATTTGTT, Rev: TGGGATAATGTATGGGAGAATG). The PCR amplicon was then denatured slowly and reannealed to facilitate heteroduplex formation. The reannealed amplicon was then digested with 5 units of T7 endonuclease I (New England Biolabs) at 37°C for 45 minutes. The samples were resolved by electrophoresis through a 3.0% agarose gel and visualized by ethidium bromide staining.

Western blot
Whole embryo protein lysates were collected at 2 dpf by homogenizing anesthetized embryos immersed in RIPA Buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X 100, protease inhibitor (Roche, cat. no. 11697498001)). 100 mg protein was loaded into individual wells of a Mini-PROTEAN TGX Precast Gel (Bio-Rad) and a western blot was performed as described [59]. Blots were incubated overnight at 4°C with anti-APOL1 antibody (1:1000; Abcam, EPR2907, ab108315). The membranes were subsequently washed in PBST (0.1% Tween 20) and incubated for 1 hour at room temperature with anti-rabbit IgG conjugated to horseradish peroxidase (1:20,000; GE Healthcare, NA934V). ACTIN antibody (1:1000, Santa-Cruz, cat. no. sc-8432) was used as a loading control.

Ethics statement
All animal protocols were reviewed and approved by the Duke University Institutional Animal Care & Use Committee (IACUC; protocol A229-12-08).

Supporting Information
S1 Fig. Complementation of zebrafish apoll 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 apoll MO and scored for edema at 5 dpf. Ectopic expression of each of the other members of the human APOL gene cluster was unable to rescue significantly the edema formation in developing embryos co-injected with apoll MO. (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. (TIF)

S2 Fig. Characterization of APOL1 protein levels in apoll-MO and APOL1 RNA-injected embryos. Protein lysates from zebrafish embryos injected with apoll-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 apoll-MO display a significant reduction in APOL1 protein expression compared to non-injected controls, suggesting cross-reactivity with zebrafish APOL1 and efficiency of the apoll 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, apoll-MO injected; Lane 3, apoll-MO + wild-type APOL1 human mRNA; Lane 4, apoll-MO + G1
APOL1 human mRNA; Lane 5, apoll-MO + G2 APOL1 human mRNA. *p = 0.026.

(PNG)

S3 Fig. myh9 suppression and complementation in developing zebrafish embryos. We recapitulated data reported by Müller et al. for experimental comparison [17]. (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.

(TIF)

S4 Fig. Further characterization of apoll and myh9 morphant glomerular ultrastructure. Transmission electron microscopy of zebrafish larval glomeruli injected with either (A) apoll-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, apoll 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 apoll-MO injected larvae, while apoll morphants appear to have a higher degree of podocyte effacement compared to myh9 morphants. (C) Zebrafish larvae injected with apoll CRISPR/CAS9 display a similar glomerular ultrastructure compared to apoll morphants at 5 dpf. Filled arrowheads, glomerular basement membrane. Scale bar = 500nm.

(TIF)

S5 Fig. Glomerular ultrastructure of apoll morphants complemented with human risk alleles. Transmission electron microscopy of zebrafish larval glomeruli imaged at 5 dpf. (A, B) apoll morphants complemented with risk alleles, G1 and G2 do not rescue the observed defects caused by apoll suppression, with naked patches of glomerular basement membrane and microvillus processes apparent. *, microvillus protrusions; filled arrowheads, glomerular basement membrane. Scale bars, 500nm.

(TIF)

S6 Fig. Complementation of apoll and myh9 morphants with each respective reciprocal human wild-type mRNA. (A) apoll-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 apoll-MO/MYH9 RNA and n = 32–46 embryos/injection for myh9-MO/APOL1 RNA); each repeated three times.

(TIF)

S7 Fig. apoll/APOL1 modulation effect on causal familial Focal Segmental Glomerulosclerosis (FSGS) genes. Zebrafish embryos were injected with either apoll-MO (1.0ng/ml 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. (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.

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Author Contributions

Concepted and designed the experiments: BRA NK MJT EED AEAK. Performed the experiments: BRA R AK SM E G. Analyzed the data: BRA AM E GE E DA AE AK. Contributed reagents/materials/analysis tools: DNH NK MJT EED AEAK. Wrote the paper: BRA DNH NK MJT EED AEAK.

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