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TITLE: APOL1 Oligomerization as the Key Mediator of
Kidney Disease in African Americans

PRINCIPAL INVESTIGATOR: Dr. Martin Pollak

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

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14. ABSTRACT The work we are conducting is aimed at understanding, and eventually preventing and treating, kidney disease, in particular the APOL1-associated form of kidney disease that accounts for the high rate of kidney disease in African Americans. This work is based on the hypothesis that APOL1 kidney disease in African Americans results from abnormal aggregation of the APOL1 risk variant protein in an amyloid-like process. We are testing this hypothesis in <i>in vitro</i> systems, cells, model organisms, and human kidney biopsy specimens using molecular biology, biochemistry, protein chemistry, and microscopy-based approaches.					
15. SUBJECT TERMS Kidney, ESRD, APOL1, African American					
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1. Introduction: The work we are conducting is aimed at understanding, and eventually preventing and treating, kidney disease, in particular the APOL1-associated form of kidney disease that accounts for the high rate of kidney disease in African Americans. This work is based on the hypothesis that APOL1 kidney disease in African Americans results from abnormal aggregation of the APOL1 risk variant protein in an amyloid-like process. We are testing this hypothesis in *in vitro* systems, cells, model organisms, and human kidney biopsy specimens using molecular biology, biochemistry, protein chemistry, and microscopy-based approaches.

2. Keywords: Kidney, ESRD, APOL1, African American

3. Accomplishments

Major goals of the project:

1: Characterize APOL1 toxic oligomer formation in cell-free and cell-based systems

We will determine whether risk variant APOL1 oligomer formation causes death of HEK293 cells by correlating the appearance of aggregates with cytotoxicity, confirming that intracellular A11 aggregates contain ApoL1 protein, and testing whether aggregation inhibitors or clearance of aggregates prevents cell death. Then we will compare the oligomerization process *in vitro* between WT and risk variant APOL1 to begin understanding how the aggregation process occurs.

2: Define the functional domains that govern APOL1 multimerization

We will further refine our understanding of the aggregation process in risk variant APOL1 by characterizing the relative affinities of different ApoL1 variants for each other. We will map first the domains and then the specific amino acids that permit APOL1-APOL1 binding. We will use this information about sites of APOL1-APOL1 binding to design and test peptide aggregation inhibitors.

3: Compare the propensity of wild-type and risk mutant APOL1 to form toxic oligomers in model systems and human kidney biopsies.

We will test for risk variant APOL1 oligomer formation in model systems of increasing complexity. We will first simulate viral infection in human cells of different APOL1 genotype with interferon stimulation, then look for oligomer formation in our APOL1 transgenic zebrafish and mouse models, and lastly test human kidney biopsy samples of different APOL1 genotype and disease phenotypes for APOL1 oligomers. This aim will directly connect APOL1 oligomers with human kidney disease and also validate the use of these animal models for *in vivo* testing of new therapies.

Accomplishments/Activities (year 1):

Overview:

We have made significant progress on our initial Aims as described below. Our work on year 1 closely follows our statement-of-work with two modifications. First, we have encountered more variation than expected from batch to batch of our A11 antibody that specifically recognizes toxic oligomers with beta sheet structure. Three different distributors produce this antibody and we have devoted significant time to clarifying the optimal antibody to use because it is central to our Aims. As we address this experimental challenge, we have substituted experiments using bioinformatics tools to identify amyloidogenic amino acids in the APOL1 sequence, mutate these amino acids, and then determine the effect of these mutations on APOL1-induced cell death. When we have resolved our difficulties with the A11 antibody, we will also test whether alterations in cell death driven by these amino acid substitutions also alter toxic oligomer or fibril formation.

Detailed discussion by task and sub-task:

Subtask 1.1A1.

Perform a time course study of APOL1 expressing cells (at 4, 8, 12, 16, 24, and 48 hours) and examine

- aggregate formation
- cell death

We have performed cell death assays to quantify the relative toxicity of APOL1 variants (G0, G1, and G2) in two systems: a transient transfection system and a tet-inducible stable expression system, both in HEK293 cells. We have found that cell death in the transient transfection system begins at 24 hours for the risk variants G1 and G2 but does not occur in G0-transfected cells until 48 hours (where G1 and G2 toxicity are still much more pronounced). In tet-induced APOL1 expressing cells of different genotypes, we have observed that cell death due to the risk variants begins at 16 hours and is very large at 24 hours (Figure 1). Risk variant cell death in this system typically exceeds G0-induced cell death by 5- to 50-fold. A representative figure is shown below [fig 1]. We have attempted to define aggregate formation in both of these systems to determine whether cell death is preceded by aggregate formation. To date our experiments are equivocal, attributed to the antibody problems described above. We will clarify this question pending optimization of our A11 antibody performance.

ApoL1 Specific Cell Death Assay

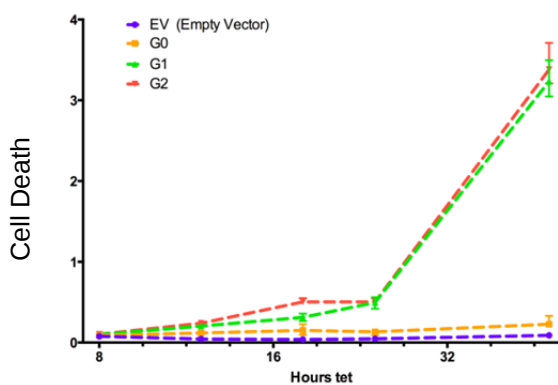


Figure 1. Timecourse of cell death (measured by cytotoxicity to viability ratio) for stably-transfected tet-inducible APOL1 expressing cells of different APOL1 genotypes.

Subtask 1.1A3. Performs assays of cell lysates to look at aggregate formation using anti-A11 antibody

- a. dot blot assays
- b. non-denaturing Western blots

Timeframe: Months 1-12

We have attempted the dot blot assay using anti-A11 antibody multiple times using transiently transfected HEK293 cells (transfected with G0, G1, or G2 APOL1 constructs). Dot blot assays did not show consistent differences in A11 positivity based on APOL1 genotype. We will repeat this assay pending A11 antibody optimization. We have observed evidence of APOL1 aggregation, especially in G2 expressing cell lines, in non-denaturing western blots using anti-APOL1 antibody, an assay that will be repeated with anti-A11 antibody.

Subtask 1.1A5. Perform immunoprecipitation

- a. precipitate A11 positive structures from cell lysates
- b. immunoblot for APOL1

Timeframe: Months 13-36

We overexpressed APOL1 risk variants G0, G1, and G2 transiently in HEK293 cells, immunoprecipitated with anti-A11 antibody, and blotted with anti-APOL1 antibody. Initial experiments demonstrated strong evidence that aggregates pulled down by anti-A11 Ab were composed of APOL1 protein, indicating that APOL1 was forming more toxic oligomers in risk variant transfected cells (G1, G2) than WT cells (G0) (Figure 2). We were unable to consistently demonstrate this result with new lots of anti-A11 antibody, a failure that directly lead to our efforts to obtain new anti-A11 antibody. This experiment is critical in testing the central hypothesis of our proposal and it will be a high priority going forward when we have reliable antibody.

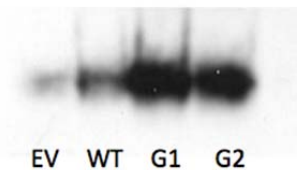


Figure 2. Lysates from APOL1-expressing cells of different APOL1 genotypes were immunoprecipitated with anti-A11 antibody and then blotted with anti-APOL1 antibody. The risk variant (G1 and G2) expressing cells have more A11-positivity, and this A11 positivity appears to be composed of APOL1 molecules.

Subtask 1.1B1: Test effect of compounds that block toxic oligomer formation on

- a. aggregate formation
- b. cell death

Compounds tested will include:

- thioflavin T, Azure C, Basic Blue 41, hemin, vanillin, daunomycin HCl, meclocycline suflosalicylate, Congo Red, Chicago Sky Blue 6B, ammonium bromides, norapomorphine, hematin, Phenol Red, rifamycin, rolitetracycline, homotaurine, myo- inositol, mannitol

Timeframe: Months 13-36

We have tested two of the above-mentioned compounds (thioflavinT, Congo Red) to determine the ability of known aggregation inhibitors to prevent APOL1-induced cell death in our stable, tet-inducible APOL1 expression system in HEK293 cells. A figure summarizing our data is shown below (figure 3). We find that the amyloidogenic dye thioflavin T blocks APOL1 induced cell death. Experiments to determine the effect of these agents on toxic oligomer formation will follow. These experiments and data from other projects suggested that APOL1 might be acting as an amyloid pore in the cell membrane. Zinc tends to be effective in blocking amyloid pores, and we determined that zinc was highly efficient at preventing cell death in this system.

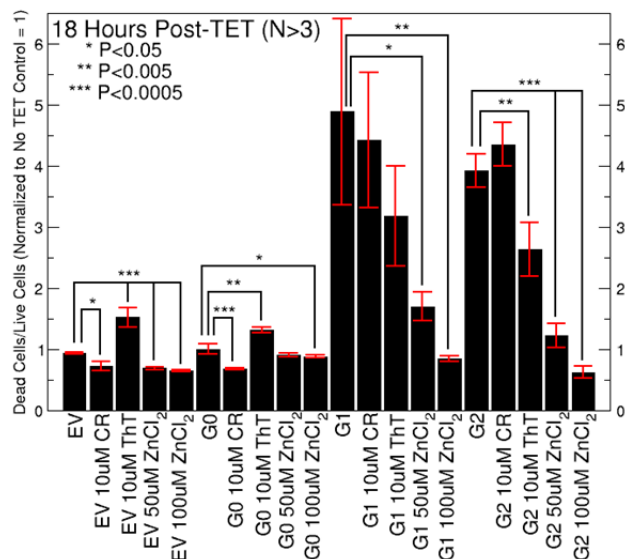


Figure 3. Thioflavin T blocks cell death induced by APOL1 overexpression. Zinc also block cytotoxicity induced by APOL1 overexpression.

Subtask 1.1B2. Test role of autophagy in aggregate formation and clearance

a. Measure (1) aggregate formation and (2) cell death after autophagy gene knockdown in cells

i. ATG5

ii. ATG7

iii. Beclin

Timeframe: Months 1-24

Preliminary experiments performed in transiently transfected HEK293 cells using shRNA knockdown of key autophagy genes did not have a significant impact cell death in our system. We interpret this data as showing that APOL1 is not causing autophagic cell death. This result correlates well with results from a broader inquiry into the APOL1 cell death mechanism in related projects.

Subtask 3.1

We will treat cells with interferons (IFN α , β , or γ)

i. treat endothelial cells with IFN

a. repeat with cells representing all combinations of APOL1 genotypes

b. stain with A11 and OC antibodies (24, 48 hours)

Timeframe: Months 7-18

We treated G0/G0 and G1/G1 human umbilical vein endothelial cells with interferon and stained for anti-oligomer (A11) antibodies. We observed positivity in both genotypes that appeared to be greater in G1/G1 cells than G0/G0 cells (shown below in figure 4). We used primary cell lines that differ at many loci beside APOL1. We plan return to this experiment using Crispr-altered cell lines generated from the same original line that differ only at the APOL1 locus.

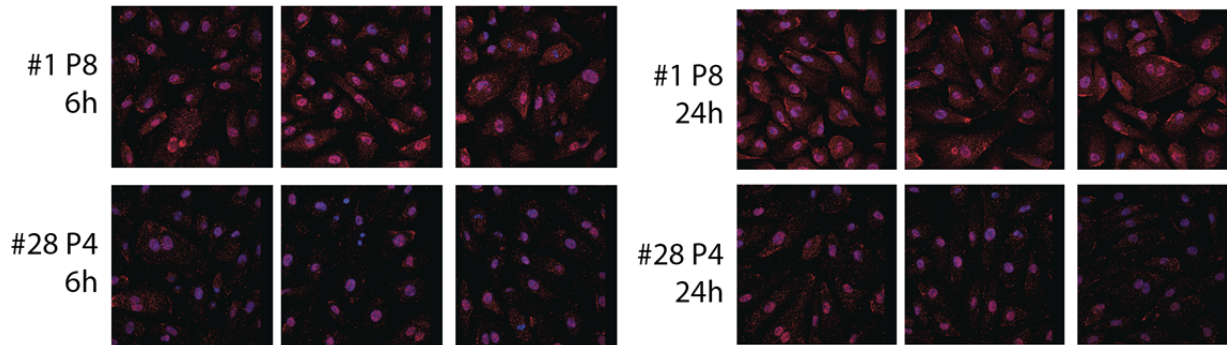


Figure 4. Cell line #1 (G1/G1) and Cell line #28 (G0/G0) were treated with interferon, which causes APOL1 levels to rise by 1-2 logfold. More A11 positivity is seen at 6 and 24 hours in the cells with the G1/G1 genotype.

Subtask 2.1.B.

Perform competitive pull down experiments

- a. Test a of each APOL1 (G0, G1, G2) with itself and with other variants
- b. Determine relative binding of the different forms

Immunoprecipitation experiments using ApoL1-G0 and its variants test the binding of each ApoL1 (G0, G1 and G2) with itself and with other variants. Results show each ApoL1 can bind with itself and other variants with different affinities (Figure 5). Then we choose SILAC experiments to quantify these affinities.

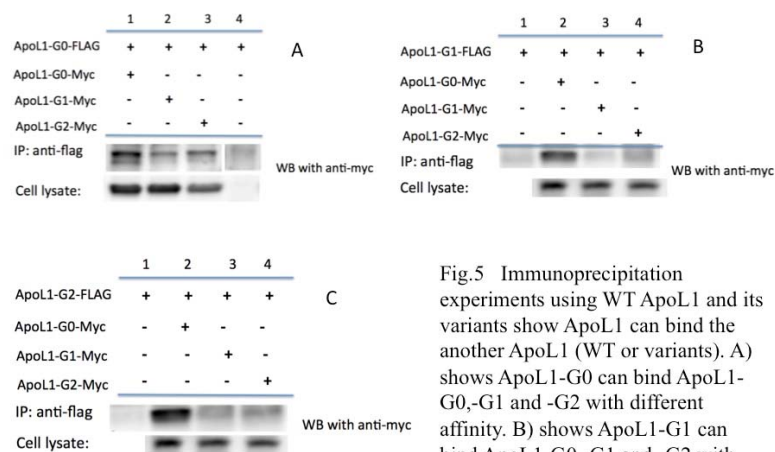


Fig.5 Immunoprecipitation experiments using WT ApoL1 and its variants show ApoL1 can bind the another ApoL1 (WT or variants). A) shows ApoL1-G0 can bind ApoL1-G0,-G1 and -G2 with different affinity. B) shows ApoL1-G1 can bind ApoL1-G0,-G1 and -G2 with different affinity. C) shows ApoL1-G0 can bind ApoL1-G0,-G1 and -G2 with different affinity.

In SILAC (stable isotope labeling by amino acids in cell culture) experiments, media with different isotopes of amino acids was used to label the different variants of ApoL1.

i.e. in Fig 6B. L, Light medium refers to normal environmental isotopes, labeled ApoL1-G1 with Flag tag; And M, for Medium medium, [$^{13}\text{C}_6$]arginine (R6) and 4,4,5,5-D4-lysine (K4), labeled ApoL1-G1 with myc tag. H, for Heavy medium, [$^{13}\text{C}_6$, $^{15}\text{N}_4$] arginine (R10) and [$^{13}\text{C}_6$, $^{15}\text{N}_2$]lysine (K8), labeled ApoL1-G0 with myc tag. ApoL1 was pulled down by anti-flag beads after we mixed these three labeled ApoL1-transfected cell lysate together. MS-LC was used to identify the proteins in the pull-down samples. In Fig 2B, ratio M/L ApoL1-G1-G1-G0 is higher than ratio H/L ApoL1-G1-G1-G0 and the value of $\log_{10}(\text{ratio H/M ApoL1-G1-G1-G0})$ is -0.112 which is out of the range of [-0.1, 0.1] indicating the binding affinity of ApoL1-G1 with itself is stronger than with ApoL1-G0.

In Fig 6A, L-medium labeled ApoL1-G0 with Flag tag, M-labeled ApoL1-G0 with myc tag, and H-labeled ApoL1-G0 with myc tag were mixed. The ratio M/L ApoL1-G0-G0-G1 is higher than the ratio H/L ApoL1-G0-G0-G1 and the value of $\log_{10}(\text{ratio H/M ApoL1-G0-G0-G1})$ is -0.21 which is out of the range of [-0.08, 0.08] showing the binding affinity of ApoL1-G0 with itself is stronger than with ApoL1-G1.

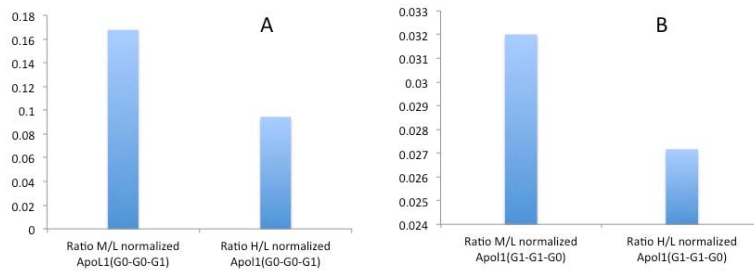


Fig.6 Stable isotope labeling by amino acids in cell culture(SILAC) experiments show the different affinity of ApoL1 binding. In A), ratio M/L is higher than ratio H/L shows the binding affinity of ApoL1-G0 with itself is stronger than with ApoL1-G1. In B), ratio M/L is higher than ratio H/L shows the binding affinity of ApoL1-G1 with itself is stronger than with ApoL1-G0. L, Light medium refers to normal environmental isotopes of carbon, nitrogen, and hydrogen, i.e. “unlabeled” ^{12}C , ^{14}N , and ^1H , whereas medium and heavy media refer to cells grown in medium containing heavy isotope-labeled arginine (R) and lysine (K) as follows: M, for medium, $^{13}\text{C}_6$ arginine (R6) and 4,4,5,5-D4-lysine (K4); H, for heavy, $^{13}\text{C}_6$, $^{15}\text{N}_4$ arginine (R10) and $^{13}\text{C}_6$, $^{15}\text{N}_2$ lysine (K8).

Perform Western blots using non-denaturing gels

Use combinations of ApoL1 holoprotein allelic variants and:

- look for differences in the size of the bands that may reflect monomers, dimers, trimers, or higher order oligomeric complexes.

We transfected ApoL1-G0, ApoL1-G1 and ApoL1-G2 into HEK 293T cells. Western blot using non-denaturing gels shows ApoL1 forms dimers; no monomers or trimers were detected (Figure 7).



Figure 7. Western blots using non-denaturing gels shows ApoL1 forms dimer.

Subtask 2.1.B.

Perform competitive pull down experiments

- b. Determine if N-N terminus binding outcompetes N-C terminus binding
- c. Determine if C-C terminus binding outcompetes N-C terminus binding

We found that for WT APOL1 there was clear evidence of N-terminal to N-terminal binding, C-terminal to C-terminal binding, and N-terminal to C-terminal binding (Figure 5,A and B) when we co-transfect plasmids encoding these variants into cells. The binding affinities between different variants of ApoL1 show a difference from each other.(Figures 8,9)

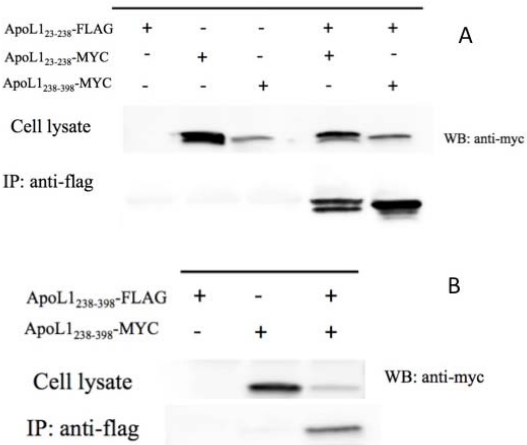


Figure 8. Immunoprecipitation experiments using WT ApoL1 show that the N-terminus of ApoL1 can bind the N- or C-terminus of another ApoL1 Molecule(A). B) indicate that the C-terminus of WT ApoL1 can similarly bind to either terminus of another ApoL1 molecule.

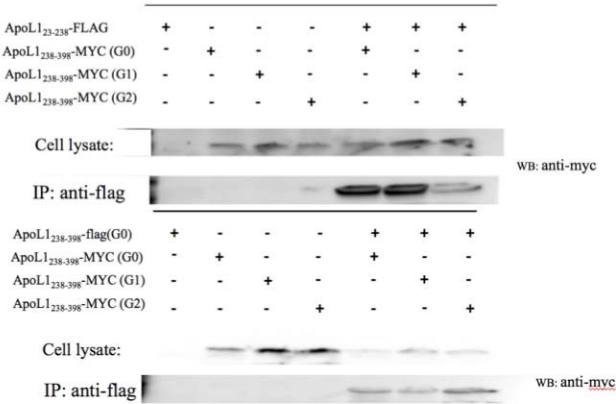


Figure 9. Immunoprecipitation experiments using different parts ApoL1 and its variants show N-terminus of ApoL1 has different affinity with variants of C-terminus and C-terminus of ApoL1 (G0) also has different affinity with variants of C-terminus of ApoL1.

Additional studies directly addressing the hypothesis of this grant but not previously described in the statement-of-work:

We have taken a parallel approach to understanding APOL1 aggregate formation using bioinformatics and mutagenesis. We have tested the APOL1 amino acid sequence in multiple algorithms that predict amyloidogenic stretches of amino acids. Two major regions are predicted to be susceptible to amyloid formation, one at the N-terminus and one near the C-terminus that includes the site of the G1 substitution:

Algorithm (G0)	Hot spot 1 (AA)	Hot spot 2 (AA)
Aggrescan	341-356	1-25
FISH	342-355	10-23
Fold Amyloid	342-354	5-23
PASTA 2.0	341-354	6-25
TANGO	341-354	9-25
WALTZ	347-356	11-23
Zygggregator	344-353	6-25

Figure 10. Amyloidogenic hotspots determined by bioinformatics analysis of APOL1 sequence.

We have begun eliminating pro-amyloidogenic amino acids in the risk variants and adding pro-amyloidogenic amino acids in WT APOL1 to determine the effect on cell death (and later on aggregate formation). Preliminary results are shown below (figure 11). After a large set of substitutions, we have identified a single pro-amyloidogenic amino acid (F343) that appears to be essential for risk variant induced cell death. Preliminary molecular modeling suggests that this substitution alters local helix integrity. This C-terminal amyloidogenic stretch of amino acids near S342G will likely be the initial candidate for in vitro investigation in **Major Task 1.2**.

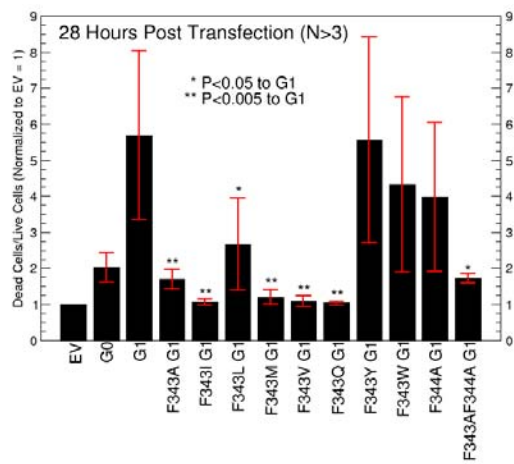


Figure 11. Cell death assays showing that changing a highly amyloidogenic amino acid (F343) to an alanine eliminates APOL1-induced cytotoxicity, whereas changing it to another amyloidogenic amino acid (tyrosine or tryptophan) does not alter APOL1 cytotoxicity.

Opportunities for training:

This project was not intended to provide training and professional development opportunities. Opportunities for professional development are provided to postdoctoral scientists through the Harvard Medical School Office for Postdoctoral Fellows.

Dissemination of results:

Dr. Pollak and Friedman have presented aspects of this work at several national meetings related to kidney disease

Plans for next reporting period:

1. (Friedman lab) Characterize APOL1 in solution for light absorbance in presence of Thioflavin T
 - a. Test both (1) APOL1 holoprotein and (2) amyloidogenic ApoL1 fragments
 - b. Repeat, varying concentration, time, pH, and temperature.
 - c. Compare WT with G1, G2 risk variants
 - d. Perform assays with mixtures of WT, G1, G2.
2. (Friedman lab) Directly visualize aggregates with atomic force microscopy (AFM) to observe differences in the size, shape, and porosity of APOL1 multimers. We will visualize three different sample preparations:
 - a. synthetic peptide fragments of APOL1
 - b. in-vitro translated APOL1
 - c. purified human APOL1.
3. (Pollak lab) Perform Western blots using non-denaturing gels. We will use combinations of ApoL1 holoprotein allelic variants and:
 - a. look for differences in the size of the bands that may reflect monomers, dimers, trimers, or higher order oligomeric complexes.
 - b. perform competition experiments to determine whether isolated ApoL1 termini can disrupt larger complex formation
4. (Friedman lab) Construct TAT-fusion constructs that act as cell-penetrating peptides
 - a. Use peptide synthesis fusion peptides (TAT cell-penetrating domain)
 - b. Purify peptides to >95% by HPLC
 - c. Confirm by mass spectroscopy.
5. (Pollak lab) Test for oligomer and fibril formation in zebrafish models of APOL1-kidney disease
 - a. stain sections of APOL1-transgenic zebrafish kidney for A11 and OC
 - b. perform double staining to for A11 or OC antibodies and ApoL1.

4. Impact

Impact on the development of the principal discipline(s) of the project?

Nothing to report

Impact on other disciplines?

Nothing to report

Impact on technology transfer?

Nothing to report

Impact on society beyond science and technology?

Nothing to report

5. Changes/Problems:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Unexpected departure of post-doctoral fellow (Dr. Lal) and delay in finding suitable replacement with biophysical skills resulted in underspending in year 1. This will be rolled forward. We do not anticipate any overall change in expenditures over the three year period of this project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. Products

Publications, conference papers, and presentations

Journal publications.

Nothing to report

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

Nothing to report

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

New DNA clones have been developed. These will be disseminated to the scientific community in response to requests once described in publications.

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. Participants

Individuals who have worked on project:

Name:	Martin R. Pollak
Project Role:	Collaborating PI
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	1.0
Contribution to Project:	As PI, overseeing all aspects of the project
Funding Support:	none

Name:	Herbert Lannon
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	2.0
Contribution to Project:	Conducting experiments to look at amyloidogenic properties of APOL1
Funding Support:	none

Name:	Ava Benjamin
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	3.0
Contribution to Project:	Assistance with biochemical and molecular biology studies
Funding Support:	none

Change in active support:

Nothing to report

Other organizations as partners:

Nothing to report