Alternative mRNA Splicing and Polyadenylation Events are Synchronous with Podocyte Injury and Slit Diaphragm Dysregulation in Glomerular Disease Models

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Short Title: Alternative Splicing and Polyadenylation in Glomerular Disease

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Abstract

Background: Glomerular disease, often characterized by podocyte injury and proteinuria, can lead to chronic kidney disease and end stage kidney disease. We hypothesized that the glomerular pathophysiology is associated with alternative mRNA processing, such as alternative splicing (AS) and polyadenylation (APA), of glomerular genes and especially of critical podocyte, slit diaphragm and matrix genes that regulate the glomerular filtration barrier.

Methods: Glomerular damage, accompanied with proteinuria, hypoalbuminemia and hypercholesterolemia was induced by puromycin aminonucleoside (PAN) or adriamycin (ADR) to mimic human minimal change disease (MCD) or focal segmental glomerulosclerosis (FSGS), respectively. Glomerular RNA-seq analyses was performed followed by JunctionSeq and APATrap bioinformatics analyses to detect AS and APA events, respectively. Novel splice sites and poly(A) usage sites and differences in alternatively spliced and polyadenylated isoforms were queried in JunctionSeq and APATrap pipelines.

Results: Altered glomerular mRNA processing by AS/APA was identified in 136/71 and 1875/746 genes in MCD and FSGS models, respectively. Transcript annotation and prioritization of significant AS and APA identified key events in genes with novel and established roles that cause disruption in the glomerular filtration barrier. These events localized in the regions of slit diaphragm (*NPHS1, NPHS2, NEPH1, ZO1, ITM2B*), glomerular basement membrane (*COL4A3/4/5, LAMB2, LAMA5*), podocyte mitochondria (*COQ2*), podocyte membrane (*PODXL*) and endothelial cells (*INF2*). Concomitantly, numerous polyadenylation and splicing factors which could serve as potential trans-factors that lead to AS and APA, were dysregulated in these two models of glomerular nephropathies. Additionally, beneficial proteinuria reducing treatment modalities, pioglitazone and GQ16 reversed many of these AS and APA events. Moreover, suggestive GWAS SNPs were identified in many of these genes from human nephrotic syndrome database, which could potentially serve as cis- factors that lead to AS and APA, and downstream functional effects.

Conclusion: Findings from our studies shed light on a new landscape of research in glomerular pathophysiology, i.e., AS and APA of glomerular genes, many of which are crucial determinants of podocyte structure and function, slit diaphragm complex, and glomerular basement membrane. These findings present new implications for improved mechanistic understanding of glomerular disease and development of novel therapeutic intervention strategies.

Significance Statement

RNA biology is complex, and while alternative mRNA processing events, such as alternative splicing (AS) and alternative polyadenylation (APA) have been shown to play important roles in physiology, development, and diseases like cancer; there is very limited knowledge of their roles in glomerular disease. Various forms of glomerular disease can unfortunately be frequently refractory to current immunosuppressive or anti-hypertensive treatments, leading to progression to chronic kidney disease and end stage kidney disease, ultimately requiring dialysis and transplant. In the current study, we identified alternative mRNA processing events of global glomerular mRNAs due to AS and APA during podocyte and glomerular injury, using two distinct animal models of glomerular disease, minimal change disease and focal segmental glomerulosclerosis. This is a newly recognized phenomenon, and it has potential implications not only for better molecular mechanistic understanding of the disease, but for identifying novel therapeutics.

Introduction

Glomerular disease is characterized by podocyte injury and loss, and manifests with high-grade proteinuria, which is accompanied by additional co-morbidities [1-6]. Various forms of glomerular disease can unfortunately be frequently refractory to existing treatments which pose additional side effects, leading to progression to chronic kidney disease (CKD) and end stage kidney disease (ESKD) [7-12]. Thus, there is a constant unmet clinical need to develop targeted therapies fueled by basic molecular studies for glomerular disease treatment.

RNAs have multifaceted functions and research and technological advances over the past decades have unraveled their true merits and remarkable complexities of their processing and various biological roles in normal cellular function and disease [13, 14]. Years of basic research has revealed that ~95% of mammalian genes undergo alternative splicing (AS), which can result in different protein products with varied functionality. Even in the absence of protein changes, AS can play a major role in determining micro-RNA sensitivity, mRNA stability, localization and translation efficiency [15, 16]. Moreover, AS is associated with many cellular processes and diseases such as differentiation, cancer, and immunity to name a few [15, 17]. While, there a few reported examples of aberrant AS in a limited number of genes relevant to CKD and glomerular disease [18], including our own work on PPARγ slice variants [19], the mechanisms and basic role of AS in podocyte biology and glomerular disease is lacking.

mRNA polyadenylation is an essential maturation step in which mRNA precursor is cleaved and trimmed at its 3' UTR and a poly(A) tail (pA) is added [20]. Changing the position of the pA site through a process called alternative polyadenylation (APA) plays an important, increasingly appreciated role in regulation of gene expression and thus in important cellular functions and pathological processes [21-29]. This has been shown to occur in ~66% of genes. Shortening of the 3' UTR can remove regulatory sequences that control RNA stability, translation, and subcellular localization [21, 22, 24, 27, 28], whereas coding region shortening can dramatically alter protein function [23, 25, 26, 29]. While global changes in APA have been observed in development and tumor progression, there are no reports on the contribution of APA in podocyte biology and glomerular disease.

Based on all of the above, we hypothesized that glomerular disease pathophysiology is interlinked with alternative processing of podocyte and glomerular mRNAs of key genes that regulate the glomerular filtration barrier. In order to study these events, we have employed animal models of glomerular disease, and identified differences in alternative splice and polyadenylated isoforms using JunctionSeq and APATrap pipelines, during disease vs healthy controls. The resulting data has yielded novel targets and deeper understanding of previously unexplored mechanisms of regulation of the genes critically involved in the functioning of the glomerular filtration barrier. Identification and mechanistic understanding of these events offers a huge potential to translate this knowledge for therapeutics and biomarkers for glomerular disease.

Methods

Animal Models

The animal studies were conducted under the approval and guidelines of the Institution Animal Care and Use Committee at Nationwide Children's Hospital. Male Wistar rats were injected intravenously (IV) with puromycin aminonucleoside (PAN) (Sigma-Aldrich, St. Louis, MO) (50mg/kg) or adriamycin (ADR) (Sigma-Aldrich) (7.5mg/kg) on Day 0 to model minimal change disease (MCD) or focal segmental glomerulosclerosis (FSGS), respectively (n=4/group). Control rats were given saline IV injections. Weights was recorded throughout the study, and spot urine and serum were collected twice a week. The PAN- treated and ADR-treated rats were euthanized on Day 11 and 21 respectively, at which time kidneys were harvested and glomeruli isolated using the sequential sieving method, as previously described [30]. Total RNA was isolated from rat glomeruli using the mirVana Isolation Kit (Life Technologies Corporation, Carlsbad, CA) according to manufacturer's instructions. Yield and purity were calculated using a nanodrop and a Qubit fluorometer. Kidney cross sections were routinely processed, sectioned at 4 µm thickness, and stained with periodic acid-Schiff method (Sigma-Aldrich). Histology was reviewed by a pathologist blinded to treatments. Equal amounts of urine (5µl) collected from rats throughout the study was resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel. Albumin bands were visualized after staining with Coomassie Brilliant Blue G-250 (Alfa Aesar, Tewksbury, MA). Urine protein:creatinine ration (UPCr) analyses were performed on final day urine by Antech Diagnostics GLP (Morrisville, NC) to quantify proteinuria. Albumin and cholesterol levels in serum were measured on the Vet Axcel (Alfa Wasserman Diagnostic Technologies, LLC, West Caldwell, NJ) using the ACE albumin and cholesterol reagents (Alfa Wasserman Diagnostic Technologies, LLC, West Caldwell, NJ) at The Ohio State University's College of Veterinary Medicine, Clinical Pathology Services, according to manufacturer's instructions.

RNASeq and Differential Expression of Genes (DEGs)

To perform RNA sequencing, mRNA libraries were generated using ~200ng total glomerular RNA (quantified using Qubit Fluorometer) with RIN of >7, using NEBNext Ultra II Directional (stranded) RNA Library Prep Kit for Illumina (NEB #E7760L), NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB #E7490) and NEBNext Multiplex Oligos for Illumina Unique Dual Index Primer Pairs (NEB #6442S/L). Sequencing of the libraries was performed with Illumina NovaSeq 6000 SP flow cell using paired-end 150-bp format (300 cycles = 2x150bp) to at least 17 million passed-filter clusters/sample (equivalent to 34 million reads) and using internal pipeline (Gadepalli et al., 2019), reads were aligned to Rat genome Rnor6.0 with HISAT2 (Kim et al., 2019) and counts generated for Rnor 6.0 v101 with featureCounts from the subread package (Liao et al., 2014). Post alignment quality check (QC) was assessed with fastqc, RseQC, and picard (Wang et al., 2012) (https://broadinstitute.github.io/picard). Counts were normalized with voom and differential expression tested with limma (Ritchie et al., 2015). Differentially expressed genes (DEGs) were chosen with adjusted $p<0.05$ and abs(logFC)>1.

JunctionSeq Analyses

For each sample, the following steps were followed for using JunctionSeq analysis [31], also outlined in http://hartleys.github.io/JunctionSeq/doc/example-walkthrough.pdf.Step 1: QoRTs QC was ran to process each sample (runQoRTsQC.pbs per sample for step 1). Step 2: QoRTs R package was used to generate QC figures and generate size factors. Step 3: QoRTs was run to make flat gff for DEXseq and JunctionSeq and to merge with novel splice sites based on samples. And finally, Step 4: JunctionSeq was used to perform differential splicing analysis and to generate plots. All samples were combined for Steps 2-4 (runJunctionSeq.pbs).

APATrap Analyses

For each sample, the following steps were followed for using APAtrap analysis [32], also outlined in https://sourceforge.net/p/apatrap/wiki/User%20Manual/. Step 1: make bedgraph (make_bedgraph.pbs). For each sample, trim raw fastq for adapters and length with trimmomatic v0.38 Map to Rnor6.0 with hisat2/2.1.0, convert to bam and sort with samtools/1.10, convert bam to bedgraph with bedtools 2.17.0 using genome CoverageBed. Step 2: identify distal 3'UTR (run_identifyDistal3UTR.pbs). With all sample bedgraphs and reference ensembl rn6 bed from UCSC, identify all 3'UTRs present in data. Step 3: predict APA (text_for_predictAPA_byCompare). With only the samples needed for a comparison and UTR bed from step 2, estimate coverage of UTRs. Limit samples to only comparison due to run time. Step 4: test differential APA (run_deAPA.R). Generate statistical test results for each measured APA for each comparison.

Heatmaps, Pathway Analysis and Ontology Enrichment

Heatmaps were generated with ComplexHeatmap in R. Enriched canonical pathways were identified using ontology enrichment performed with MOET – MultiOntology Enrichment Tool [MOET, Ontology Enrichment (mcw.edu)] to identify enriched biological processes, cellular components and molecular functions.

GWAS Correlation with AS and APA Genes

Genes that were identified in the AS and APA dataset in the MCD model were cross-referenced for GWAS SNPs identified from patients with steroid sensitive nephrotic syndrome ([33] to generate Manhattan plots. For the AS dataset, any SNP in the gene region covering the exons, introns, 5' and 3' UTRs and splice site junctions was plotted and for the APA dataset, any SNP in the 3' UTR region was plotted.

Statistics

Statistical analyses were performed and adjusted p values used as described above for RNASeq DEG, JunctionSeq and APATrap data. Differentially expressed genes (DEGs) were chosen with adjusted $p<0.05$ and abs(logFC)>1, unless otherwise noted. SNPs threshold was significant at -log10(P) value of $7.3 = 5X10^{-8}$, and suggestive at -log10(P) value $1.3 = 5X10^{-2}$.

Results

I. Puromycin Aminonucleoside (PAN) and Adriamycin (ADR)- Induced Models of Nephropathy Reveal Common and Distinct Transcriptome Signatures in Glomerulopathies

PAN and ADR- Induced Nephropathy Models in Rats Correspond to the Human Minimal Change Disease (MCD) and Focal Segmental Glomerulosclerosis (FSGS)

In order to study the alternative processing of glomerular mRNAs during disease, we used two well-established rat models of podocyte and glomerular injury; puromycin aminonucleoside - induced minimal change disease (PAN-MCD) and adriamycin-induced focal segmental glomerulosclerosis (ADR-FSGS) (**Figure 1A**). Proteinuria was induced by single IV PAN injection of 50 mg/kg to male Wistar rats (35.83 mg/mg ± SEM 16.47 mg/mg vs 2.58 mg/mg ± SEM 0.38 mg/mg; p=0.028, Day 11), which started appearing on Day 4 (**Figures 1B and C**). Massive proteinuria was observed in ADR injected (7.5 mg/kg) male Wistar rats, starting from day 7 (88.31 mg/mg ± SEM 16.95 mg/mg vs 2.58 mg/mg ± SEM 0.38 mg/mg; p=0.002, Day 21) (**Figures 1B and C**). Control rats received IV saline injection. Histologic evaluation of the kidneys revealed normal renal parenchyma in healthy controls (**Figure 1D**). In contrast, all of the ADR mice displayed multifocal dilation of proximal tubules due to the presence of intraluminal protein casts. Many of the glomeruli showed marked hypertrophy of podocytes with prominent intracytoplasmic vacuolation (**Figure 1D**). Serum chemistry profile revealed conditions like hypoalbuminemia and hypercholesterolemia and several other parameters altered in the ADR model of nephropathy (**Supplementary Figure S1**).

RNA-Seq, Ontology Enrichment Analyses, Deconvolution and Comparison with Human Monogenic Genes for Podocytopathies Reveal Unique and Shared Transcriptomic Landscape and Pathways in PAN and ADR Models

In order to gain insights into the genes and molecular pathways involved in these two disease models, we performed deep paired RNA Seq analysis from the glomeruli isolated from healthy control rats and from PAN and ADR-injected rats at the end of the study. Principal Component analysis (PCA) showed a glomerular transcriptomic shift in PAN and ADR groups compared to controls, as these groups formed distinct clusters indicating the gene expression profiles of these groups are significantly different from each other (**Figure 1E**). An overlap among the PAN and ADR clusters was also observed, suggesting some shared molecular characteristics between these groups. Glomerular injury with PAN identified a total of 1,032 DEGs (988 upregulated, 44 downregulated) compared to controls (adj $p<0.05$ and log2FC>1) (**Figure 1F**). Glomerular injury with ADR identified a total of 1,304 DEGs (1215 upregulated, 89 downregulated) compared to controls (adj $p<0.05$ and log2FC>1). Our analysis also aimed to identify and compare common and distinct upregulated and downregulated DEGs in PAN and ADR to understand the specific gene expression profiles associated with each disease model (**Figure 1G**). Ontology enrichment analysis identified the top cellular components (**Figure 1Hi**), biological processes (**Figure 1Hii**), and molecular functions (**Figure 1Hiii**) associated with each disease model for their unique DEGs and shared DEGs. These included extracellular matrix, actin cytoskeleton, intercellular bridges, IL6 and TNFα production, and components of synaptic membrane.

These were further categorized by all the DEGs in PAN and ADR models as well as DEGs that were either up- or down-regulated in these models (**Supplementary Figure S2**).

Further deconvolution of the transcriptomic data identified that the altered transcriptome contributing towards the pathogenesis in the PAN and ADR models can be attributed to genes specific to podocytes as well as other glomerular cell types, including mesangial, endothelial cells and parietal epithelial cells (PECs), including various PEC subtypes (**Supplementary Figure S3, Supplementary Table 1**). Moreover, we identified a number of established monogenic genes with known association with glomerular disease and podocytopathies as well as novel genes without any prior association in the transcriptomic data in both PAN and ADR models of nephropathy (**Supplementary Figure S4**).

II. Disease Pathogenesis in PAN and ADR Models is Associated with Glomerular mRNA Alternative Splicing (AS)

Glomerular mRNAs Undergo Alternative Splicing (AS) during Glomerular Injury

Alternative splicing (AS) of mRNA precursors provides an important means of genetic control, and its mis-regulation underlies many human diseases, however, role of alternative splicing (AS) in podocyte and glomerular biology and pathophysiology is completely lacking. We used the JunctionSeq program [31] to analyze the RNASeq dataset to identify AS mRNAs in the PAN and ADR models. Glomerular injury identified a total of 136 and 1875 AS glomerular genes compared to controls, in PAN and ADR models, respectively (adj $p<0.05$) (**Figure 2A, B, Supplementary Figure S5**). Among these, 104 genes were shared, while 32 were unique to the PAN model and 1771 unique to the ADR model.

AS Events are Specific to Podocyte Genes as well as Other Glomerular Cells (Mesangial, Parietal and Endothelial)

PAN-MCD and ADR-FSGS models are characterized by podocyte injury, although glomerular preparations are composed of other cell types as well, such as endothelial cells, mesangial cells and parietal epithelial cells, along with some infiltration from immune cells and minor contribution from proximal tubule cells. In order to understand the contribution of these other cell types in the glomeruli and podocyte specific AS events in these two models of injury, we deconvoluted the JunctionSeq-derived dataset by cross-referencing the significant AS events in PAN and ADR models with top 50 genes specifically expressed in these multiple cell types [34]. Podocyte specific genes *Nphs1, Podxl1* were identified to be AS in both disease models, while the ADR model presented with additional AS events in *Tmsb4x* and *Rhpn1* genes (**Figure 2Ci-iii**). Mesangial specific gene *Map1lc3a* was found to be AS in ADR, and a few endothelial genes were found to be AS in either both the models (*Flt1*) or uniquely in the ADR model (i.e. *Sgk1, Adgrf5, Ehd3*). Immune cell specific gene, *Msn*, was AS in both disease models, however, *Rpsa and B2m w*ere uniquely AS in ADR, indicating their specific contribution to ADR model. Very few proximal tubule cell specific genes such as *Ppp1r1a, Atp1a1, and Epcam* were identified to be AS in ADR disease model, and only *Atp1a1* in PAN model, suggesting limited contribution from the proximal tubule components. Furthermore, with the recent advancements in the diverse roles of parietal epithelial cell (PEC) sub-types in the regeneration of

podocytes and pathophysiology of glomerular injury, we further deconvoluted the JunctionSeq-derived dataset with significant AS events from these models with the variety of PEC sub-populations [35]. We identified AS events in all the five sub populations, with the most events clustered in the podocyte progenitor A1 sub population and surprisingly in the tubular progenitors A4 population, especially in the ADR model (**Figure 2Di and ii, Supplementary Table 2**). In summary, this data suggests that the AS events are distributed in the diverse glomerular cell types as well as within the PEC subpopulation during PAN and ADR glomerular injury.

Glomerular mRNAs Undergo AS in Genes with Novel and Established Roles in Glomerulopathies

In order to correlate the genes identified with AS events in the PAN and ADR glomerular injury models with the glomerular disease in human patients, we cross-referenced our dataset with 80 genes with established monogenic roles in glomerular disease [36]and 49 genes associated with podocytopathies and steroid resistant nephrotic syndrome [37]. We identified 8 AS genes in PAN model and 19 AS genes in ADR model with established monogenic roles in glomerular disease (**Figure 2E)**. These mostly localized within the podocytes (membrane, cytoskeletal scaffold, nuclear proteins and transcription factors, slit diaphragm and mitochondria), endothelial cells or glomerular basement membrane. Among these, 7 genes were common in both models, while 14 were additionally AS in the ADR model. The common genes included those localized to the podocyte membrane (*Podxl*) and cytoskeletal scaffold (*Tns2, Inf2, Itsn2*) and matrix genes in the glomerular basement membrane (GBM), such as *Lamb2* and *Col4a4*. ADR model exhibited additional genes such as *Col4a3/5* in the GBM and *Nphs1* in the slit diaphragm. Furthermore, upon comparison with genes associated with steroid resistant nephrotic syndrome, we identified 4 and 15 AS genes in PAN and ADR models, respectively (**Figure 2E**). These are involved in various podocyte functions, such as actin binding and regulation, slit membrane, and CoQ biosynthesis. In summary, our data on glomerular AS genes in the two disease models reflects upon multiple modes of regulation of genes with established monogenic roles in human glomerular disease (**Figure 2G, Supplementary Table 3**). Additionally, this study uncovered novel genes without any prior established monogenic role from human glomerular disease dataset (**Figure 2H, Supplementary Table 4**).

Furthermore, we have previously reported that the therapeutic treatment modalities, pioglitazone and GQ-16, are beneficial in reducing proteinuria in PAN model of glomerular disease [30]. We further identified that many of these glomerular AS events are reversed with Pio (60 AS events vs. PAN) and GQ16 (15 AS events vs. PAN) treatments, suggesting the crucial role these could be playing in regulating the downstream effects on podocyte health and injury (**Supplementary Tables 5, 6**).

III. Disease Pathogenesis in PAN and ADR Models is Associated with Glomerular mRNA Alternative poly(A) Site Usage and Polyadenylation (APA)

Glomerular mRNAs Undergo Alternative Polyadenylation (APA) during Glomerular Injury and a Global Shift from Proximal to Distal Poly(A) Site Usage

Alternative polyadenylation (APA) is an RNA-processing mechanism that generates distinct 3' termini on mRNAs resulting in variable stability and protein products, but role of APA in podocyte and glomerular biology and pathophysiology remains unexplored. We used the APATrap program [32] to analyze the RNASeq dataset to identify APA mRNAs in the PAN and ADR models. Glomerular injury identified a total of 71 and 746 APA glomerular genes compared to controls, in PAN and ADR models, respectively (adj $p<0.05$) (**Figure 3A**). Among these, 55 genes were shared, while 15 were unique to the PAN model and 689 unique to the ADR model. Moreover, a proximal to distal shift in poly(A) site usage was observed in 61 genes in PAN and 715 genes in ADR model, while the reverse shift from distal to proximal poly(A) site usage was observed in only 10 genes in PAN and 31 genes in the ADR model (**Figure 3Bi,ii**).

APA Events are Specific to Podocyte Genes as well as Other Glomerular Cells (Mesangial, Parietal and Endothelial)

PAN-MCD and ADR-FSGS models are characterized by podocyte injury, as well as alterations in other glomerular cell types as well. In order to understand the distribution of identified glomerular APA events in these two models of injury, we deconvoluted the APATrap derived dataset by cross-referencing the significant APA events in PAN and ADR models with top 50 genes specifically expressed in these multiple cell types [34]. Podocyte specific genes *Nphs1, Podxl1* were identified to be altered in both disease models, while the ADR model presented with additional APA events in *Nphs2* gene (**Figure 3Ci-iii**). Mesangial specific gene *Map1lc3a* and endothelial specific gene *Thbd* was found to be APA in ADR. Deconvolution of APA genes by various PEC subpopulations [35] identified APA events in mostly podocyte progenitor A1 sub population, both in PAN and ADR models (**Figure 3Di, ii, Supplementary Table 7**). In summary, these data suggest that the APA events are distributed in the diverse glomerular cell types, and particularly within the podocyte progenitor PEC-A1 subpopulation during PAN and ADR glomerular injury.

Glomerular mRNAs Undergo APA in Genes with Novel and Established Roles in Glomerulopathies, with Varied miRNA Targeted Sites

Comparison of APA dataset genes in the PAN and ADR glomerular injury models with 80 monogenic genes in glomerular disease [36]and 49 genes associated with podocytopathies and steroid resistant nephrotic syndrome [37], revealed a number of APA events in the genes with established roles in human glomerular disease. We identified 7 APA genes in PAN model and 10 APA genes in ADR model with established monogenic roles in glomerular disease (**Figure 3E)**. These mostly localized within the podocytes and in the slit diaphragm. Among these, 6 genes were common in both models (*Podxl, Myh9, Kirrel1, Nphs1, Nphs2, Cd151)*, while 4 were additionally APA in the ADR model. Interestingly, Wt1 was uniquely identified in the ADR model and *Lmx1b* in the PAN model. Furthermore, comparison with genes associated with steroid resistant nephrotic syndrome revealed 3 and 7 APA genes in PAN and ADR models, respectively (**Figure 3F**). These are involved in various podocyte functions, such as actin binding, regulation and slit membrane. In summary, our data on glomerular APA genes in the two disease models reflects upon an additional layer of regulation of genes with established roles in human glomerular disease **(Figure 3G, Supplementary Table 8)**.

Additionally, this study uncovered novel genes without any prior established monogenic roles from human glomerular disease dataset **(Figure 3H, Supplementary Table 9)**. For each APA gene, predicted miRNA sites were identified from miRNA database (miRDB.org). A target score of 74 or above was selected and at maximum ten miRs with the highest target scores were listed for each gene. For each miR, its sequence is listed as well.

Furthermore, we have previously reported that the therapeutic treatment modalities, pioglitazone and GQ-16, are beneficial in reducing proteinuria in PAN model of glomerular disease [30]. We further identified that many of these glomerular APA events (including in slit diaphragm component genes such as *Kirrel1*) are reversed with Pio (56 APA events vs. PAN) and GQ16 (20 APA events vs. PAN) treatments, suggesting the crucial role these could play in regulating the downstream effects on podocyte health and injury (**Supplementary Tables 10, 11**).

IV. *Trans-elements of mRNA Processing, such as RNA Binding Proteins, Splicing and Polyadenylation Factors, are Dysregulated in Glomerular Disease Models*

Splicing Factors and RNA Binding Proteins are Dysregulated in Glomerulopathies

RNA binding proteins (RBPs) and splicing factors play a critical role in regulating mRNA splicing during normal physiology, development and pathophysiology of many diseases, such as cancer[16]. Due to complete lack of information on their involvement and possible trans- roles in regulating AS in podocytes, we compiled a database of ~400 RBPs that play an established role in mRNA splicing from literature [38, 39] and cross-referenced with the DEGs (adj p <0.05, FC>1) in our MCD and FSGS animal model RNASeq datasets (**Figure 4Ai,ii, Supplementary Table 12**). We identified 112 (MCD) and 155 (FSGS) splicing factors that were dysregulated (mostly upregulated), suggesting that the AS observed in the glomerular genes during injury could be the result of the dysregulation of splicing factors and RBPs upstream of the splicing events. These included various class of splicing factors, such as U1/2/4/5/6/11/12 small nuclear ribonucleoproteins (snRNPs), factors recruited at A, B, and C complex, specific alternative splicing factors, and RNA binding motif (RBM) proteins. Interestingly, RBM47, was the only splicing factor that was found to be highly downregulated, specifically in the ADR model. Moreover, while there was a significant overlap in two disease models (96), a number of unique factors were dysregulated in PAN (16) and ADR (60) model.

Polyadenylation Factors are Dysregulated in Glomerulopathies

The molecular mechanisms underlying APA involve variations in the concentration of core poly(A) site cleavage, polyadenylation and processing factors and several RNA-binding proteins [40]. In order to understand the contribution of these trans-factors in APA events observed in the glomerular mRNAs during injury, we compiled a database of ~209 factors of the polyadenylation machinery from literature [40] and cross-referenced these with the DEGs (adj p <0.05, FC>1) in our MCD and FSGS animal model RNASeq datasets (**Figure 4Bi,ii, Supplementary Table 13**). We identified 39 (MCD) and 51 (FSGS) polyadenylation factors that were dysregulated (upregulated), with a significant overlap in two disease models (36), and a few factors uniquely dysregulated in PAN (3) and ADR (15) model. These factors mostly included proteins of the pre-mRNA cleavage complex, polyA factors of

cleavage and polyadenylation specificity factor (CPSF) complex and core sequence elements and factors.

Evolving research shows that the processes of mRNA transcription and processing (splicing and polyadenylation) and even translation can be intertwined events [40, 41]. Thus, we analyzed our dataset in the glomerular disease models in this study to identify dysregulated DEGs that were also represented in factors and RBPs involved in mRNA transcription and translation machinery that could be coupled with mRNA processing. We identified several factors, such as RNA polymerase II associated factors, DNA damage response factors, and translation factors that were dysregulated in both PAN (24) and ADR (28) models (**Figure 4Ci,ii, Supplementary Table 14**).

The above data combined suggest a possible mechanistic explanation of the observed AS and APA events observed in the PAN and ADR glomerular disease models.

V. Cis-Elements with a Potential to Alter mRNA Splicing and Polyadenylation were Identified as Suggestive SNPs from Genome Wide Association Studies of Glomerular Disease Patients

Evolving genome wide association studies (GWAS) has identified numerous genetic determinants or single nucleotide polymorphisms (SNPs) utilizing multi-population glomerular disease and healthy cohorts [33, 42]. Most of these genetic variants are noncoding, indicating their potential role in gene regulation. In order to understand the functional and clinical consequences of these GWAS signals, we cross-referenced the GWAS signals from multi-population study (38,463 participants, including 2440 cases) of SNPs associated with steroid sensitive nephrotic syndrome (NS) with the genomic region of 136 AS genes and 3' UTR regions of 71 APA genes in the PAN-MCD model dataset from this study [33]. We identified a few significant SNPs (e.g. *EHMT2, PRRC2A, NPHS1*), and several suggestive SNPs (e.g. T*JP1, ITM2B, PODXL, MYO1D, COL4A4, NPHS2*) in AS genes from our datasets (**Figure 5A**). We also identified a few suggestive SNPs (e.g. *NPHS1, PODXL*) in the 3' UTR regions of APA genes from our datasets (**Figure 5B**). These data suggest the possibility of SNPs associated with NS to impart their effects by altered mRNA processing (splicing/polyadenylation) of the corresponding genes.

Discussion

Current treatments for glomerular disease with immunosuppressive or anti-hypertensive agents can be frequently ineffective, leading to CKD, which effects ~10% of the world population [7-12]. Alternative therapies such as calcineurin inhibitors (cyclosporine, tacrolimus) and cytotoxic agents (cyclophosphamide) can induce remission in some of these patients, but these therapies also suffer from significant side effects [43-49]. In order to develop better non-toxic and effective therapies, it is critical to understand basic mechanisms to identify targetable therapeutic pathways. In this regard, RNA therapeutics is a fast-evolving field, and in the past decade several pre-clinical and clinical studies have guided the potential for RNA therapies to treat different diseases [50-53]. Despite these advances in RNA biology in various diseases, there is a considerable knowledge gap and unmet need to guide these concepts to the field of podocyte biology and glomerular disease. This stems from an absolute lack of understanding of mRNA processing events, such as AS and APA in the physiology and pathophysiology of podocytes and glomeruli. In the current study, we utilized two well-established models of glomerular disease for MCD and FSGS, to decipher the mRNA transcript landscape at the level of the constitution and heterogeneity of transcripts due to mRNA AS and APA, beyond the quantification of the transcript levels. We have identified several AS and APA events in these models which potentially offer critical layers of gene regulation. These kinds of events have been recognized as important steps in cellular development and in normal physiology as well as many pathological processes in other diseases [13, 14, 21-29], however, this is the first study to understand these mechanisms in podocyte biology and glomerulopathies. There are a few recently published studies highlighting the role of AS in proximal tubules in diabetic kidney disease [54], and in kidney development [55], however there is no precedence of investigating AS and APA in its entirety in glomerular disease.

Transcriptomic and the AS and APA profiles of the PAN and ADR models revealed numerous previously unrecognized transcript changes in the glomeruli, with differential contribution of podocytes, mesangial cells, endothelial cells and PEC within the glomeruli and the involvement of different PEC subtypes. Moreover, while MCD (PAN model) and FSGS (ADR model) are both podocyte and glomerular disease that are common causes of idiopathic nephrotic syndrome in pediatric and adult population, they have different disease pathologies, etiologies, disease course and treatment outcome [56, 57]. FSGS, usually being a more severe form of treatment-resistant disease, is also reflected not just in transcriptomic profiles, but in their AS and APA profiles as well. Moreover, while there are obvious distinct differences between the two models, we identified many overlapping genes, pathways and AS and APA events as well, suggesting common underlying mechanisms, and perhaps a strength to the notion that these two diseases are different manifestations of the same progressive disease [58].

Of particular interest is the identification of AS and APA events in the regions of slit diaphragm (*NPHS1, NPHS2, NEPH1, ZO1, ITM2B*) and glomerular basement membrane (*COL4A3/4/5, LAMB2, LAMA5*), which are critical components of the glomerular filtration barrier. Moreover, we identified a reversal of some of these events with treatment modalities, pioglitazone and GQ16, which we have previously demonstrated to be beneficial in reducing proteinuria and glomerular injury [30, 49, 59]. This suggests a pathogenic role of these events, in addition to an associative role, although future studies

are warranted. Additionally, we observed concomitant dysregulation of numerous polyadenylation and splicing factors in these two models of glomerular nephropathy, which may provide trans-regulatory effects on AS and APA. Moreover, suggestive GWAS SNPs were identified in many of these genes from human nephrotic syndrome database, which could potentially serve as cis- factors that lead to AS and APA, and downstream functional effects.

There are various strengths and limitations to our study. While, we have utilized well-established models of glomerular disease which mimic human MCD and FSGS remarkably well [60], and we have identified critical components of the slit diaphragm and filtration barrier that exhibit AS and APA events, further validation of these targets in human biopsies will provide confirmation. However, these studies are not straightforward, as in addition to high cost, they require precise probes and fresh samples to be able to capture the differential variants. Another critical barrier is the use of time-consuming bioinformatics approach to read, align and stitch segments of exons, junctions and 3'UTRs to arrive at the potential targets. While we have utilized very effective and proven approaches [31, 32], newly emerging technologies using long read sequencing and poly(A)-ClickSeq could provide more definitive, less laborious approaches to identify AS and APA events. These approaches are, however, not cost-effective, lack enough depth in terms of million reads per transcript to arrive at significant differences between the groups, and were not easily accessible at the time this study was initiated. In contrast, our studies were designed to generate >34 million reads/sample which were analyzed by the latest established bioinformatics software [31, 32], thus providing a rich and robust dataset to identify targets. Moreover, the approach used in this study has the potential to identify numerous previously non-annotated splice site junctions and transcripts. However, we also recognize that due to the split nature of transcript reads across the entire length of the gene, the targets will need to be validated and confirmed for the AS and APA transcripts using conventional PCR techniques. Another limitation is the use of bulk RNASeq data on the glomeruli which is not effective in delineating cell-specific AS and APA events. However, the use of scRNASeq doesn't provide enough depth and is limited to typically capturing only the 3' or 5' end of the polyadenylated transcripts in the final sequencing library. This allows for the quantification of gene expression, but omits the feasibility of AS or APA analysis [61]. We have successfully deconvoluted the RNASeq data to specific glomerular cell types as well as PEC subtypes based on the cell-specific signatures available from glomerular scRNA Seq databases [34, 35].

In summary, the analyses of alternative mRNA splicing and polyadenylation in MCD and FSGS models in the present study provide novel insights and mechanisms of the pathophysiology of these glomerulopathies. We have identified several AS and APA events in genes which are crucial determinants of podocyte structure and function, slit diaphragm complex, and glomerular basement membrane. These findings present new implications for improved mechanistic understanding of glomerular disease and development of novel therapeutic intervention strategies.

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Author Contributions

MKD performed experiments, analyzed and interpreted the data, prepared figures and tables, and drafted parts of the manuscript. AW performed bioinformatics analyses, prepared figures and tables, and drafted parts of the manuscript. MY, CY and CB performed experiments and prepared figures and tables. RG and CM interpreted the data and edited the manuscript. SA conceptualized and designed the study, analyzed and interpreted the data, prepared figures and tables, and drafted and edited the manuscript. All the authors approve of the final version of the manuscript. Part of these findings were presented at the 14th International Podocyte Conference, held at University of Pennsylvania, Philadelphia, PA in 2023 and at the ASN-Kidney Week held in Philadelphia, PA in 2023.

Conflicts of Interest

None

Figure Legends

Figure 1

Puromycin amino-nucleoside (PAN)- and adriamycin (ADR)- induced models of nephropathy reveal common and distinct transcriptome signatures in glomerulopathies. **(A)** Schematic for PAN and ADR-induced nephropathy models showing a single IV injection of **(Ai)** PAN (50 mg/kg) and **(Aii)** ADR (7.5 mg/kg) in male Wistar rats resulted in **(B)** induction of proteinuria and increased urinary protein/creatinine ratio (UPCr) compared to healthy controls. **(C)** Representative urine gels showing temporal increase in albumin bands in both PAN and ADR models. **(D)** Periodic acid schiff (PAS) stained images (10X and 40X) of the renal cortex of a representative rat from each group: **(Di)** control, **(Dii)** PAN- induced nephropathy, and **(Diii)** ADR- induced nephropathy. At the observed timepoints there are no visible histologic changes in the PAN model (Day 11) **(ii)**. However, in the ADR model (Day 21), there are many dilated proximal tubules which contain protein casts throughout the cortex and the glomeruli have hypertrophied podocytes with vacuolated cytoplasm **(iii)**. At least 10 glomerular fields were observed per mouse (n=4) per group. Scale bar 50 µm, 40X images and 100 µm, 10X images. **(E)** RNA Seq followed by Principal Component analysis (PCA) depicting a glomerular transcriptomic shift in PAN and ADR groups compared to controls. Each dot represents a rat. **(F)** Venn diagram of differentially expressed genes (DEGs) observed in the glomeruli of PAN and ADR models compared to healthy controls. 840 DEGs are common, 192 DEGs unique to PAN model, and 464 DEGs unique to ADR model. **(G)** Common and distinct upregulated and downregulated DEGs in PAN and ADR disease models. 805 genes are upregulated and 35 genes are downregulated in both PAN and ADR. 183 and 410 genes were distinctly upregulated, and 9 and 54 genes were distinctly downregulated in the PAN and ADR models, respectively. **(H)** Ontology enrichment analysis identified the top cellular components (GOCC, **Hi**), biological processes (GOBP, **Hii**), and molecular functions (GOMF, **Hiii**) associated with each disease model for their unique DEGs and shared DEGs. (**Hi**) The cellular components of DEGs unique to ADR included extracellular matrix and collagen trimer, those unique to PAN included basement membrane and synaptic membrane and of DEGs shared between the two models included actin cytoskeleton and intercellular bridges. (**Hii**) The biological processes identified from the DEGs unique to ADR included extracellular matrix and collagen fibril organization and IL6 and TNFα production, those unique to PAN included regulation of nuclear division and chromosome segregation and of DEGs shared between the two models included extracellular matrix organization and collagen biosynthesis. (**Hiii**) The molecular functions identified from the DEGs unique to ADR included extracellular matrix binding and of DEGs shared between the two models included actin, integrin and microtubule binding.

Figure 2

Glomerular mRNAs are alternatively spliced (AS) in puromycin amino-nucleoside (PAN)- and adriamycin (ADR)- induced nephropathy models mimicking MCD and FSGS. **(A)** Schematic showing the experimental and bioinformatic workflow to detect AS mRNA transcripts (shown as mutually exclusive exons) in the glomeruli of rats subjected to PAN- and ADR-induced injury. AS transcripts

can also be generated due to additional splicing events (such as alternative 5' and 3' splice sites usage, intron retention, exon skipping/inclusion) not depicted in the schematic. Venn diagram depicts the common and distinct AS genes detected in both the models compared to healthy rats. **(B)** MA plots depict the relationship between normalized coverage and fold change between groups highlighting the measures that are much higher in one group than the other, observed in **(Bi)** PAN and **(Bii)** ADR models. **(C)** Deconvolution distribution of AS genes across the different glomerular cell types, including podocytes, mesangial cells, endothelial cells and a small fraction of immune cells and proximal tubule cells, in **(Ci)** PAN and **(Cii)** ADR models. **(Ciii)** Genes from the deconvolution represented in PAN and ADR models. **(D)** Deconvolution distribution of AS genes across the different parietal epithelial cell (PEC) subtypes, including PEC-A1, PEC-A2, PEC-A3, PEC-A4 and PEC-B population, in **(Di)** PAN and **(Dii)** ADR models. AS genes in PAN and ADR models represented in **(E)** monogenic glomerular disease genes, and **(F)** podocyte genes associated with steroid resistance nephrotic syndrome. AS genes in PAN and ADR models represented in **(G)** genes with established roles in glomerular disease, and **(H)** genes with novel roles in glomerular disease.

Figure 3

Glomerular mRNAs are alternatively polyadenylated (APA) in puromycin amino-nucleoside (PAN)- and adriamycin (ADR)- induced nephropathy models mimicking MCD and FSGS. **(Ai)** Schematic showing the experimental and bioinformatic workflow to detect APA mRNA transcripts in the glomeruli of rats subjected to PAN- and ADR-induced injury. Venn diagram depicts the common and distinct APA genes detected in both the models compared to healthy rats. **(B)** Volcano plots depict the 3'UTR length difference and a shift towards distal vs proximal poly A site usage for each of the significant APA genes observed in the **(Bi)** PAN and **(Bii)** ADR model. **(C)** Deconvolution distribution of APA genes across the different glomerular cell types, including podocytes, mesangial cells, endothelial cells and a small fraction of immune cells and proximal tubule cells, in **(Ci)** PAN and **(Cii)** ADR models. **(Ciii)** Genes from the deconvolution represented in PAN and ADR models. **(D)** Deconvolution distribution of APA genes across the different parietal epithelial cell (PEC) subtypes, including PEC-A1, PEC-A2, PEC-A3, PEC-A4 and PEC-B population, in **(Di)** PAN and **(Dii)** ADR models. APA genes in PAN and ADR models represented in **(E)** monogenic glomerular disease genes, and **(F)** podocyte genes associated with steroid resistance nephrotic syndrome. APA genes in PAN and ADR models represented in **(G)** genes with established roles in glomerular disease, and **(H)** genes with novel roles in glomerular disease.

Figure 4

A multitude of splicing and polyadenylation factors, RNA binding proteins and transcription and translation machinery factors are dysregulated in the PAN and ADR models of glomerular disease. **(A)** Heatmap of DEGs in the PAN and ADR models represented in the dataset of ~400 splicing factors and RNA binding proteins (compiled for this study) that play an established role in mRNA splicing. **(i)** Cross-referenced with the DEGs (adj p <0.05, FC>1) in the PAN RNASeq dataset, **(ii)** Cross-referenced with the DEGs (adj p <0.05, FC>1) in the ADR RNASeq dataset. **(B)** Heatmap of DEGs in the PAN and ADR models represented in the dataset of ~209 polyadenylation factors (compiled for

this study), that play an established role in mRNA polyA site cleavage and polyadenation. **(i)** Cross-referenced with the DEGs (adj p <0.05, FC>1) in the PAN RNASeq dataset, **(ii)** Cross-referenced with the DEGs (adj p <0.05, FC>1) in the ADR RNASeq dataset. **(C)** Heatmap of DEGs in the PAN and ADR models represented in the dataset of a combination of transcription and translation machinery factors (compiled for this study). **(i)** Cross-referenced with the DEGs (adj p <0.05, FC>1) in the PAN RNASeq dataset, **(ii)** Cross-referenced with the DEGs (adj p <0.05, FC>1) in the ADR RNASeq dataset.

Figure 5

Manhattan plots depicting multi-population suggestive GWAS signals/SNPs localized in the regulatory regions of AS genes and in the 3'UTR of APA genes in the PAN model. **(A)** Genomic regions (including introns, exons, 5'UTR, 3'UTR, and splice sit junctions) in the 136 AS genes and **(B)** 3' UTR regions in the 71 APA genes, from the datasets in the PAN model, were cross-referenced with the GWAS signals from multi-population study of SNPs associated with steroid sensitive nephrotic syndrome. These regions were defined by the grch37 to match the GWAS version. Genes of interest are marked with an arrow. SNPs threshold; Significant: -log10(P) value of 7.3 = $5X10^{-8}$, above the red line; Suggestive: -log10(P) value 1.3 = $5X10^{-2}$, above the blue line.

Supplementary Files:

Supplementary Figure S1
Serum chemistry of PAN- and ADR- induced nephropathy animal models
Supplementary Figure S2
Ontology enrichment analyses of DEGs in PAN- and ADR- induced nephropathy models
Supplementary Figure S3
DEGs in PAN and ADR models deconvoluted for glomerular cells and PEC subtypes
Supplementary Figure S4
DEGs in PAN and ADR models represented in monogenic genes for glomerular disease and podocytopathies
Supplementary Table 1
Deconvolution of DEGs in PAN and ADR models for PEC subtypes
Supplementary Table 2
Deconvolution of AS genes in PAN and ADR models for PEC subtypes
Supplementary Table 3
AS genes in PAN and ADR models represented in monogenic genes with established roles in glomerular disease
Supplementary Table 4
AS genes in PAN and ADR models representing novel genes in glomerular disease
Supplementary Table 5
AS genes reversed with pioglitazone treatment
Supplementary Table 6
AS genes reversed with GQ-16 treatment
Supplementary Table 7
Deconvolution of APA genes in PAN and ADR models for PEC subtypes
Supplementary Table 8
APA genes in PAN and ADR models represented in monogenic genes with established roles in glomerular disease
Supplementary Table 9
APA genes in PAN and ADR models representing novel genes in glomerular disease
Supplementary Table 10
APA genes reversed with pioglitazone treatment
Supplementary Table 11
APA genes reversed with GQ-16 treatment
Supplementary Table 12
Dysregulated splicing factors in PAN and ADR models
Supplementary Table 13
Dysregulated polyadenylation factors in PAN and ADR models
Supplementary Table 14
Dysregulated transcription and translation machinery factors in PAN and ADR models

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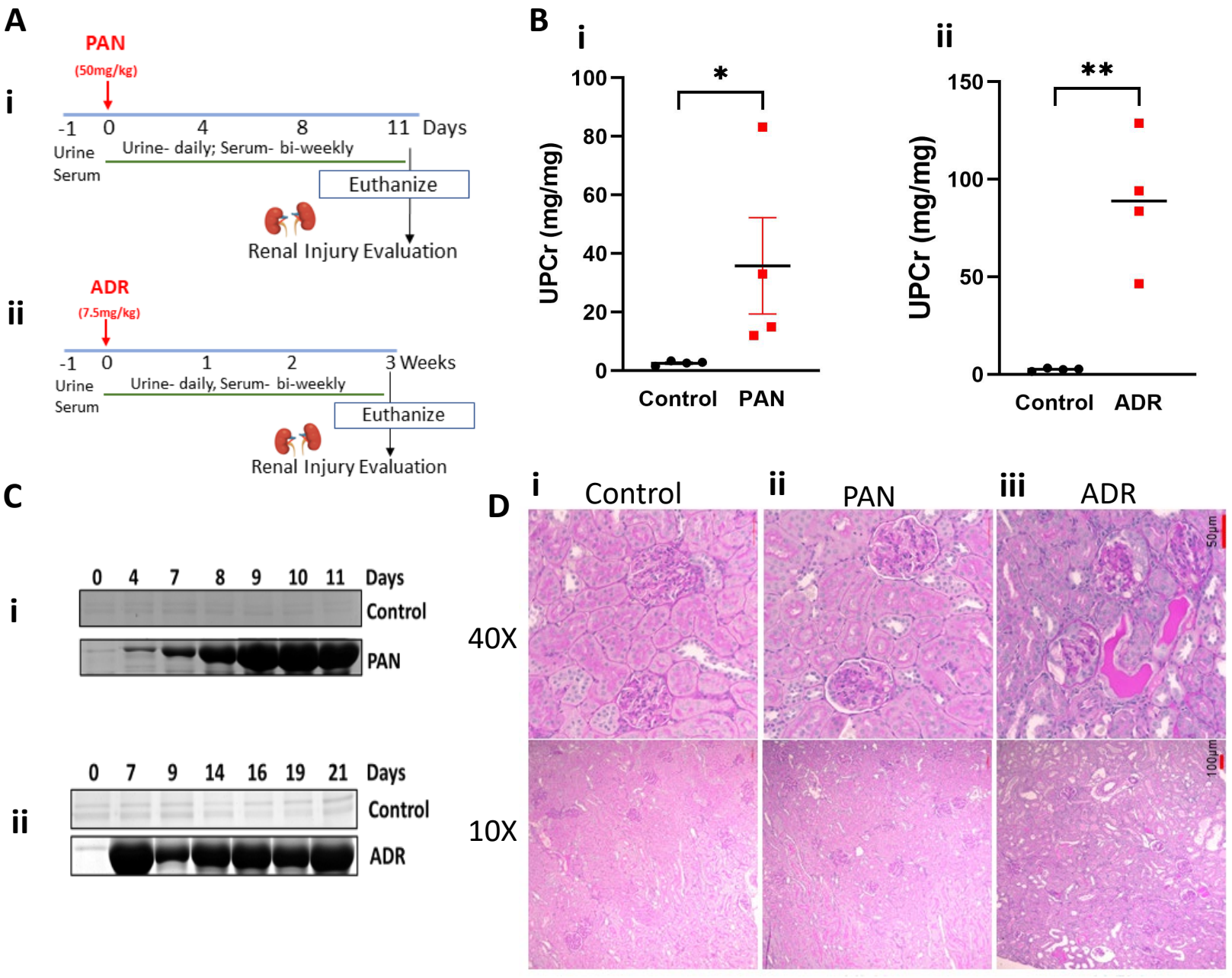

A
i
PAN
(50mg/kg)
-1 0 4 8 11 Days
Urine
Serum
Urine- daily; Serum- bi-weekly
Euthanize
Renal Injury Evaluation
ii
ADR
(7.5mg/kg)
-1 0 1 2 3 Weeks
Urine
Serum
Urine- daily, Serum- bi-weekly
Euthanize
Renal Injury Evaluation
B
i
UPCr (mg/mg)
100
80
60
40
20
0
*
Control
PAN
ii
UPCr (mg/mg)
150
100
50
0
**
Control
ADR
C
i
0 4 7 8 9 10 11 Days
Control
PAN
ii
0 7 9 14 16 19 21 Days
Control
ADR
D
i Control
ii PAN
iii ADR
40X
10X
50µm
100µm

Figure 1

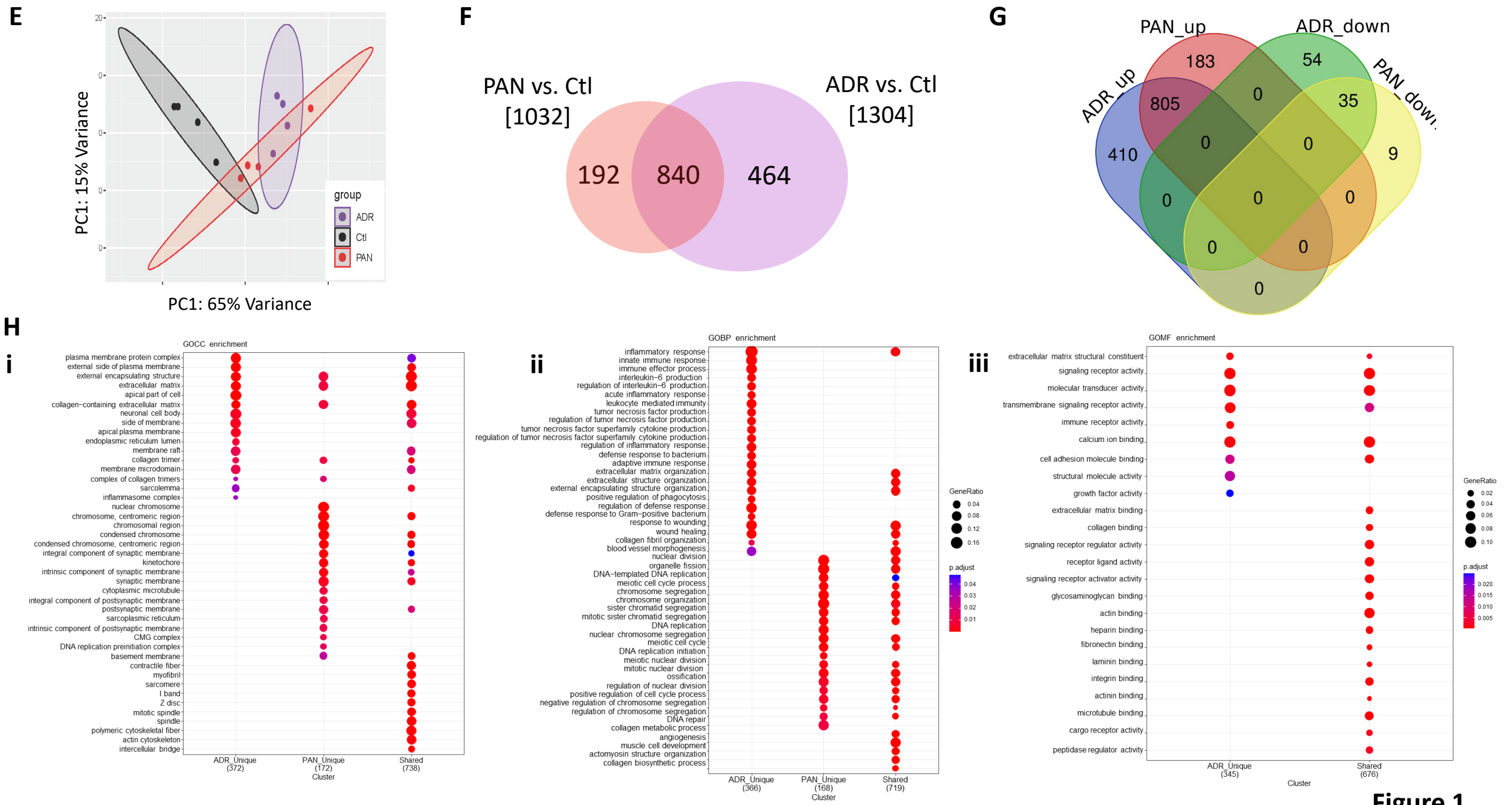

E
PC1: 15% Variance
PC1: 65% Variance
group
ADR
Ctl
PAN
F
PAN vs. Ctl
[1032]
ADR vs. Ctl
[1304]
192
840
464
G
PAN_up
ADR_down
ADR_up
PAN_down
183
54
805
35
410
9
0
H
i
GOCC enrichment
plasma membrane protein complex
external side of plasma membrane
external encapsulating structure
extracellular matrix
apical part of cell
collagen-containing extracellular matrix
neuronal cell body
side of membrane
apical plasma membrane
endoplasmic reticulum lumen
membrane raft
collagen trimer
membrane microdomain
complex of collagen trimers
sarcolemma
inflammasome complex
nuclear chromosome
chromosome, centromeric region
chromosomal region
condensed chromosome
condensed chromosome, centromeric region
integral component of synaptic membrane
kinetochore
intrinsic component of synaptic membrane
synaptic membrane
cytoplasmic microtubule
integral component of postsynaptic membrane
postsynaptic membrane
sarcoplasmic reticulum
intrinsic component of postsynaptic membrane
CMG complex
DNA replication preinitiation complex
basement membrane
contractile fiber
myofibril
sarcomere
I band
Z disc
mitotic spindle
spindle
polymeric cytoskeletal fiber
actin cytoskeleton
intercellular bridge
ADR_Unique (372)
PAN_Unique (172)
Shared (738)
Cluster
ii
GOBP enrichment
inflammatory response
innate immune response
immune effector process
interleukin-6 production
regulation of interleukin-6 production
acute inflammatory response
leukocyte mediated immunity
tumor necrosis factor production
regulation of tumor necrosis factor production
tumor necrosis factor superfamily cytokine production
regulation of tumor necrosis factor superfamily cytokine production
regulation of inflammatory response
defense response to bacterium
adaptive immune response
extracellular matrix organization
extracellular structure organization
external encapsulating structure organization
positive regulation of phagocytosis
regulation of defense response
defense response to Gram-positive bacterium
response to wounding
wound healing
collagen fibril organization
blood vessel morphogenesis
nuclear division
organelle fission
DNA-templated DNA replication
meiotic cell cycle process
chromosome segregation
chromosome organization
sister chromatid segregation
mitotic sister chromatid segregation
DNA replication
nuclear chromosome segregation
meiotic cell cycle
DNA replication initiation
meiotic nuclear division
mitotic nuclear division
ossification
regulation of nuclear division
positive regulation of cell cycle process
negative regulation of chromosome segregation
regulation of chromosome segregation
DNA repair
collagen metabolic process
angiogenesis
muscle cell development
actomyosin structure organization
collagen biosynthetic process
ADR_Unique (366)
PAN_Unique (168)
Shared (719)
Cluster
GeneRatio
0.04
0.08
0.12
0.16
p.adjust
0.04
0.03
0.02
0.01
iii
GOMF enrichment
extracellular matrix structural constituent
signaling receptor activity
molecular transducer activity
transmembrane signaling receptor activity
immune receptor activity
calcium ion binding
cell adhesion molecule binding
structural molecule activity
growth factor activity
extracellular matrix binding
collagen binding
signaling receptor regulator activity
receptor ligand activity
signaling receptor activator activity
glycosaminoglycan binding
actin binding
heparin binding
fibronectin binding
laminin binding
integrin binding
actinin binding
microtubule binding
cargo receptor activity
peptidase regulator activity
ADR_Unique (345)
Shared (676)
Cluster
GeneRatio
0.02
0.04
0.06
0.08
0.10
p.adjust
0.020
0.015
0.010
0.005

Figure 1

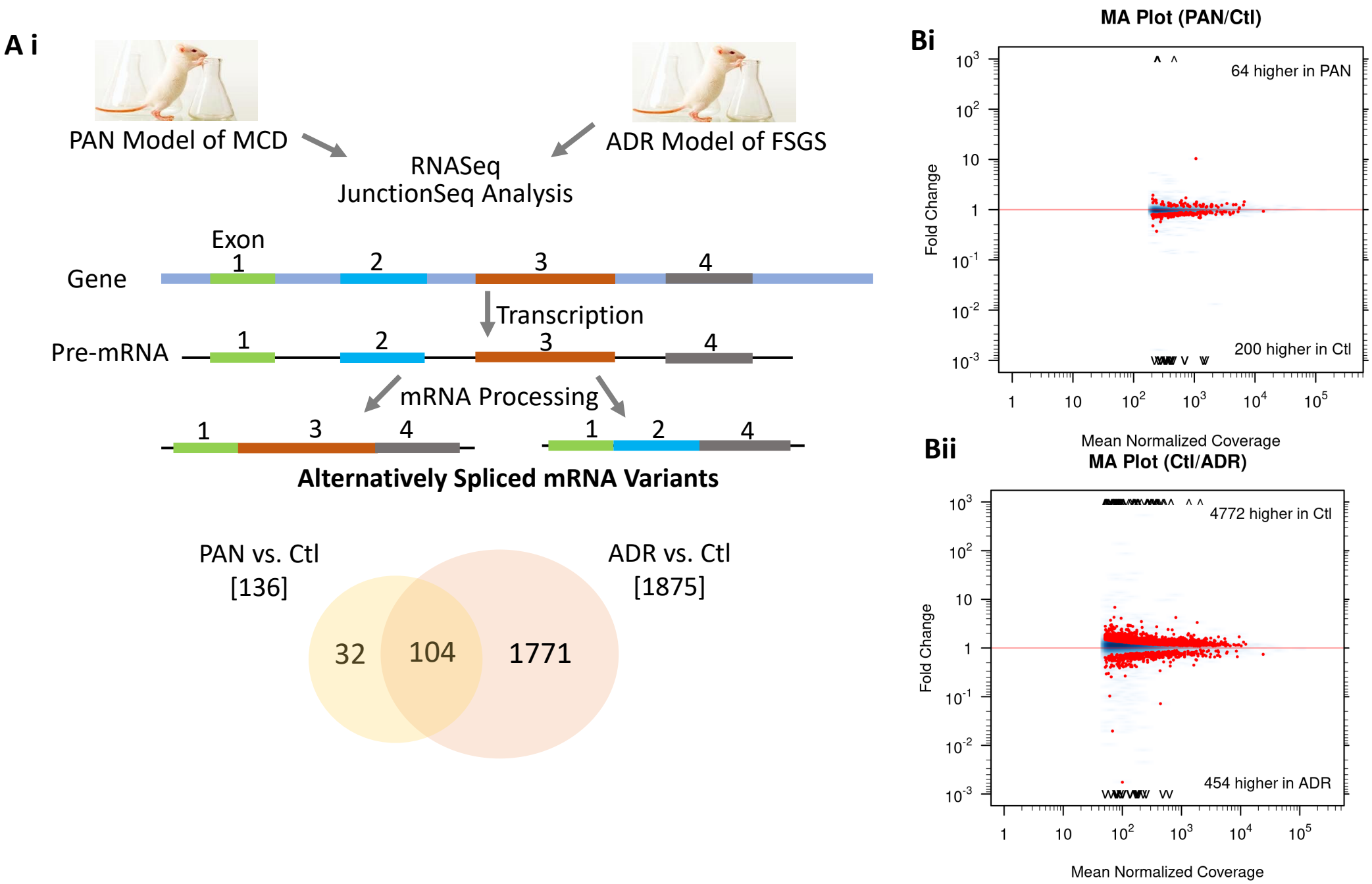
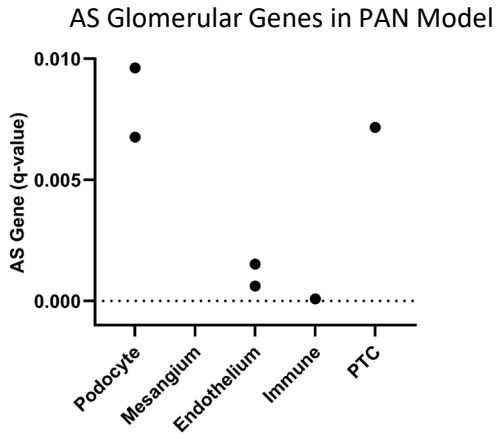

A i
PAN Model of MCD
ADR Model of FSGS
RNASeq
JunctionSeq Analysis
Exon
Gene
1
2
3
4
Transcription
Pre-mRNA
mRNA Processing
Alternatively Spliced mRNA Variants
PAN vs. Ctl
[136]
ADR vs. Ctl
[1875]
32
104
1771
Bi
MA Plot (PAN/Ctl)
64 higher in PAN
200 higher in Ctl
Fold Change
Mean Normalized Coverage
Bii
MA Plot (Ctl/ADR)
4772 higher in Ctl
454 higher in ADR
Fold Change
Mean Normalized Coverage

Figure 2

C

i



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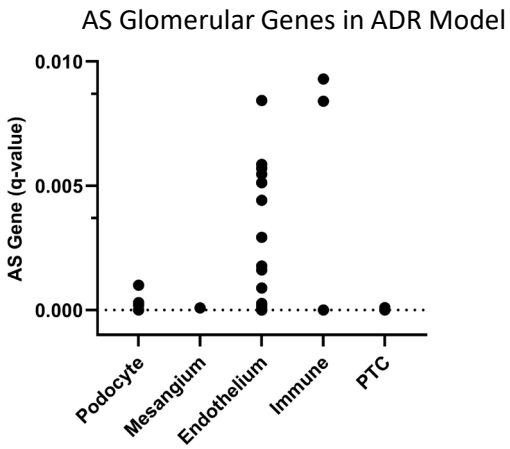


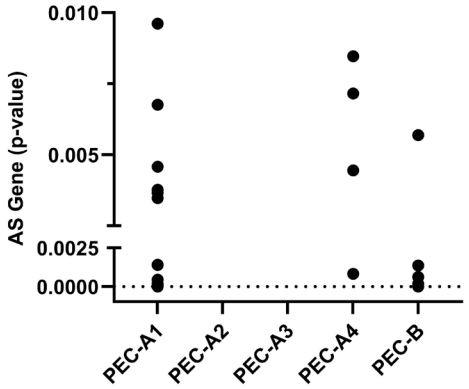
iii

Deconvolution of AS genes in PAN and ADR Models for Glomerular Cell Population

Cell types	PAN	ADR
Podocyte	*Nphs1, Podxl*	*Tmsb4x, Nphs1, Podxl, Rhpn1*
Mesangium		*Map1lc3a*
Endothelium	*Flt1, Plpp1*	*Adgrf5, B2m, Calcrl, Clic4, Ednrb, Ehd3, Emp1, Flt1, Fxyd5, Gimap6, Igfbp5, Ly6e, Mat2a, Pi16, Plpp1, Plpp3, Ptprb, Sgk1, Slfn5*
Immune	*Msn*	*B2m, Msn, Rpsa*
PTC	*Atp1a1*	*Atp1a1, Ppp1r1a, Epcam*

D

i



ii

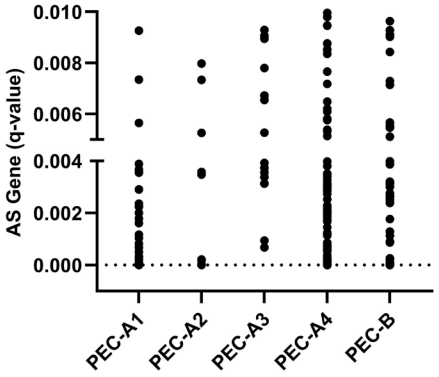


Figure 2

E

Monogenic Glomerular Disease Genes Represented in the AS Genes in PAN and ADR Models			
Class	**Glomerulus**	**PAN (MCD)**	**ADR (FSGS)**
Podocytes	Membrane	*Podxl*	*Podxl*
	Cytoskeletal Scaffold	*Tns2, Inf2, Itsn2*	*Tns2, Inf2, Dlc1, Myh9, Myo1e*
	Lysosomal and endocytic		*Scarb2*
	Nucleoporins and transcription factors	*Lmna*	*Lmna*
	Slit Diaphragm		*Nphs1, Fat1*
	Metabolic and cystolic		*Ankfy1*
	Mitochondrial		*Coq2, Pdss2*
	Endothelial cells	*Inf2*	*Inf2*
Matrix	GBM	*Lamb2, Col4a4*	*Lamb2, Lama5, Col4a3, Col4a4, Col4a5*

F

Podocyte Genes Associated with Steroid Resistance Represented in the AS Genes in PAN and ADR Models		
Class	**PAN (MCD)**	**ADR (FSGS)**
Actin Binding	*Inf2, Lamb2*	*Lamb2, Myh9, Myo1e, Inf2*
Slit membrane	*Nphs1, Nphs2*	*Fat1, Nphs1*
Lysosome		*Scarb2*
tRNA modification		*Cubn, Lage3*
Actin Regulation		*Kank2, Rac1, Dlc1, Cdc42*
CoQ biosynthesis		*Coq2, Pdss2*

Figure 2

G

Alternative Spliced Genes with Established Roles in Glomerular Disease			
Gene	**Protein**	**PAN**	**ADR**
Coq2	CoenzymeQ2 / Polypolyprenyl transferase		3.77e-4
Pdss2	Decaprenyl Diphosphate Synthase Subunit 2		4.76e-3
Ankfy1	Ankyrin repeat and FYVE domain-containing protein 1		1.99e-4
Dlc1	Rho GTPase-activating protein 7		1.09e-4
Myh9	Myosin-9		5.05e-3
Tns2	Tenisn 2	2.73e-3	2.26e-4
Inf2	Inverted Formin 2	3.59e-3	3.45e-4
Itsn2	Intersectin 2	5.68e-3	
Podxl	Podocalyxin	6.76e-3	3.24e-4
Scarb2	Scavenger receptor class B member 2 or lysosomal integral membrane protein-2 (LIMP-2)		9.63e-3
Lmna	lamin A and C	5.59e-3	2.35e-3
Fat1	FAT1 cadherin protein		2.16e-4
Nphs1	Nephrin	9.62e-3	1.99e-4
Nphs2	Podocin	3.48e-3	
Myo1e	Myosin 1e		2.92e-4
Rac1	Rac Family Small GTPase 1		6.5e-3
Cdc42	Cell division control protein42		1.22e-3
Cubn	Cubilin		5.21e-3
Lage3	L antigen family member 3		4.63e-4
Col4a3	Collagen IV alpha 3		8.43e-5
Col4a4	Collagen IV alpha 4	1.71e-4	2.09e-4
Col4a5	Collagen IV alpha 5		9.37e-3
Lama5	Laminin-a5		5.73e-6
Lamb2	laminin-b2	2.88e-3	2.17e-3

H

Alternative Spliced Genes with Novel Roles in Glomerular Disease			
Gene	**Protein**	**PAN**	**ADR**
Itm2b	Integral Transmembrane Protein 2B	1.05e-4	6.2e-26
Adgrg1	Adhesion G Protein-Coupled Receptor G1	---	5.01e-19
Polr1a	RNA Polymerase I Subunit A	---	3.96e-15
Tas1r2	Taste receptor type 1 member 2	---	6.42e-11
Tacc2	Transforming Acidic Coiled-Coil Containing Protein 2	4.32e-3	1.66e-10
Rgs3	Regulator of G Protein Signaling 3	1.33e-7	7.18e-10
Cab39	Calcium Binding Protein 39	---	2.82e-3
Tjp1	Tight Junction Protein 1	1.38e-4	2.74e-3
Lima1	LIM Domain and Actin Binding 1	---	6.41e-9
Ampd2	Adenosine Monophosphate Deaminase 2	---	8.27e-9
Macf1	Microtubule-actin crosslinking factor 1	9.91e-5	1.79e-8
Cwc27	Spliceosome Associated Cyclophilin	---	2.34e-8

Figure 2

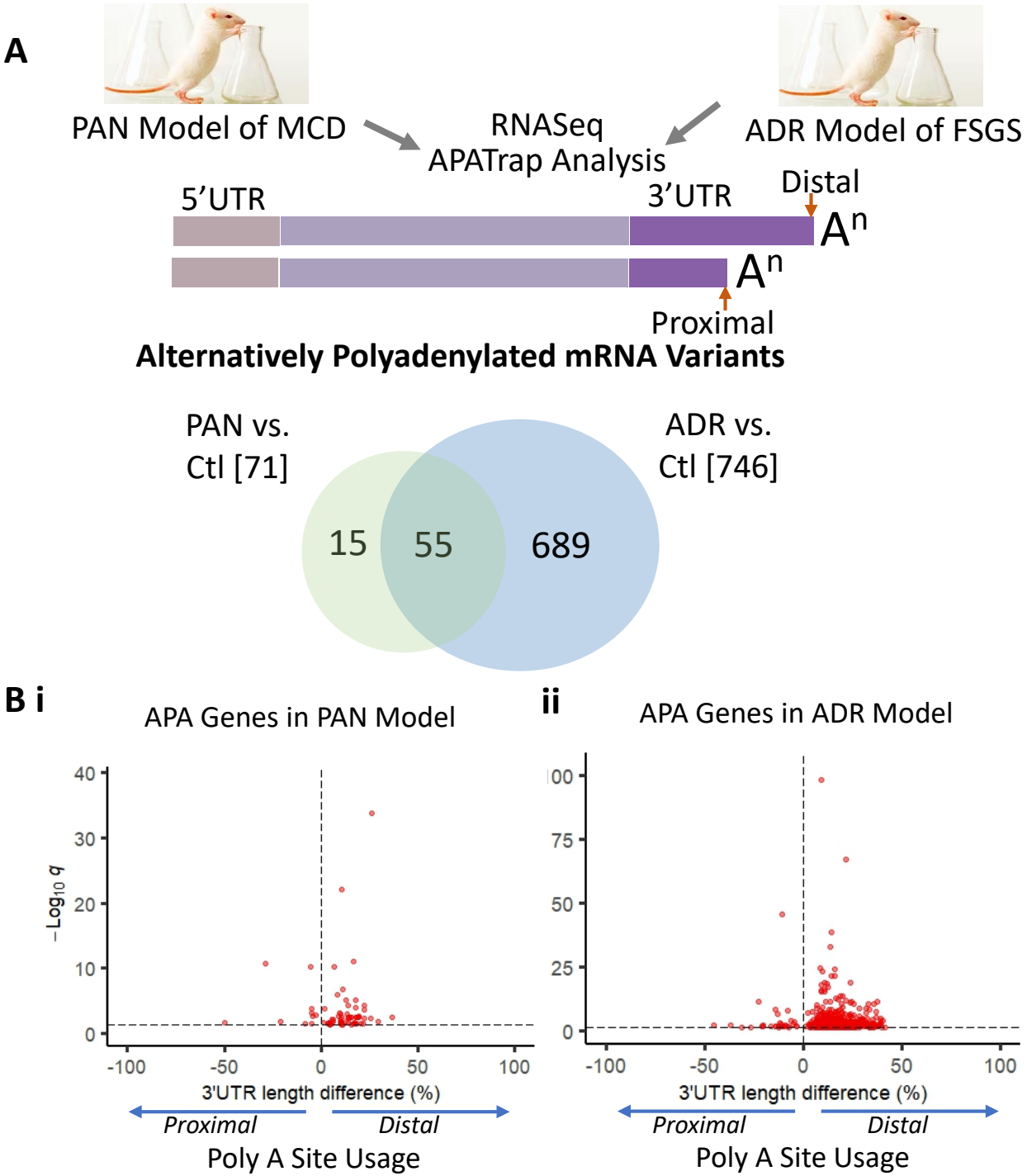
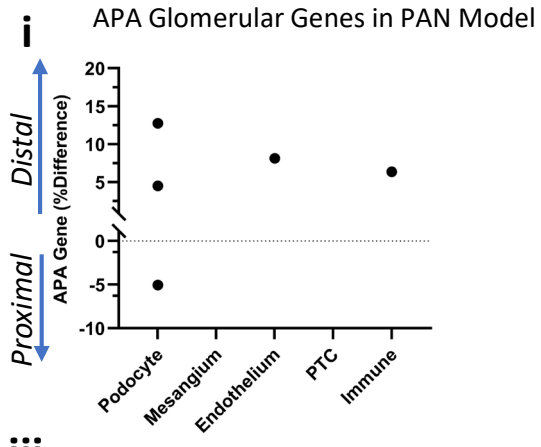

A
PAN Model of MCD
RNASeq
APATrap Analysis
ADR Model of FSGS
5'UTR
3'UTR
Distal
Proximal
Alternatively Polyadenylated mRNA Variants
PAN vs.
Ctl [71]
ADR vs.
Ctl [746]
15
55
689
B i
APA Genes in PAN Model
ii
APA Genes in ADR Model
−Log10 q
3'UTR length difference (%)
Proximal
Distal
Poly A Site Usage

Figure 3

C

i



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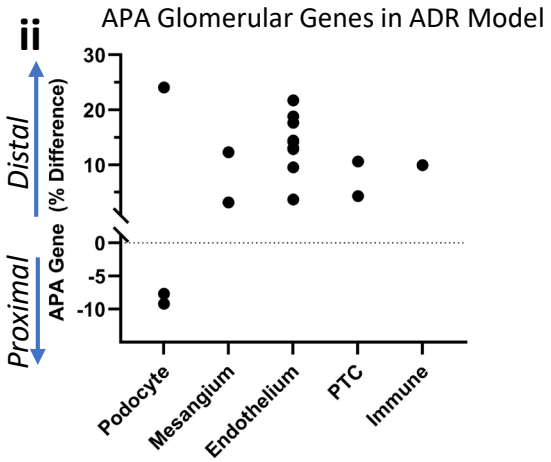


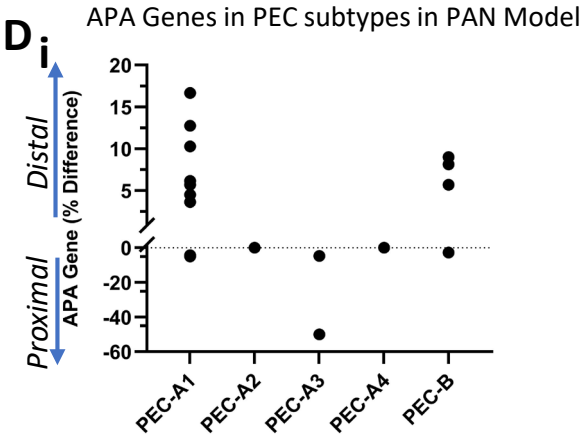
iii

Deconvolution APA genes in PAN and ADR Models for Glomerular Cell Population

Cell types	PAN	ADR
Podocyte	*Nphs1, Podxl, Rhpn1*	*Nphs2, Nphs1, Podxl*
Mesangium		*Mgp, Map1lc3a*
Endothelium	*Igfbp5*	*Nrp1, Ptprb, Sgk1, Igfbp5, Mat2a, Ednrb, Clic4, Thbd, Gpx1, Plpp3*
Immune	*Msn*	*Msn*
PTC		*Atp1a1, Epcam*

D

i



ii

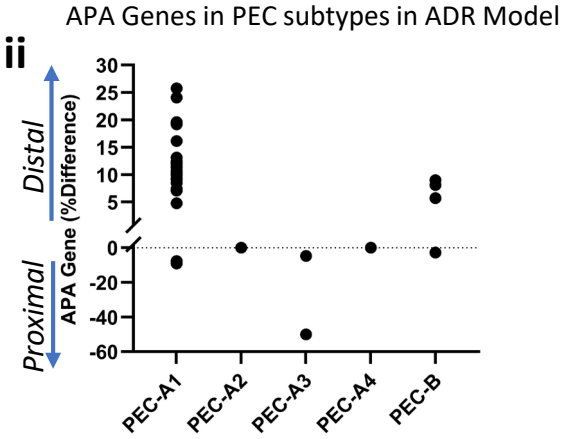


Figure 3

E

Monogenic Glomerular Disease Genes Represented in the APA Genes in PAN and ADR Models			
Class	**Glomerulus**	**PAN (MCD)**	**ADR (FSGS)**
Podocytes	Membrane	*Podxl*	*Podxl*
	Cytoskeletal Scaffold	*Myh9*	*Myh9, Dlc1*
	Lysosomal and endocytic		*Scarb2*
	Nucleoporins and transcription factors	*Lmx1b*	*Wt1*
	Slit Diaphragm	*Kirrel1, Nphs1, Nphs2*	*Kirrel1, Nphs1, Nphs2*
	Endothelial cells	---	*Thbd*
Matrix	GBM	*Cd151*	*Cd151*

F

Podocyte Genes Associated with Steroid Resistance Represented in the APA Genes in PAN and ADR Models		
Class	**PAN (MCD)**	**ADR (FSGS)**
Actin Binding	*Myh9*	*Myh9*
Slit membrane	*Nphs1, Nphs2*	*Nphs1, Nphs2*
Lysosome		*Scarb2*
S1P metabolism		*Sgpl1*
Actin Regulation		*Rac1, Dlc1*

Figure 3

G

Alternatively Polyadenylated Genes with Established Roles in Glomerular Disease			
Genes	**Protein**	PAN (%)	ADR (%)
Podxl	Podocalyxin	50.45(P)	7.68 (P)
Myh9	Myosin-9	4.39 (D)	10.94 (D)
Dlc1	Rho GTPase-activating protein 7		13.41 (D)
Scarb2	Scavenger receptor class B member 2		19.95 (D)
Wt1	WT1 Transcription factor		10.31 (D)
Kirrel1	Kirre like nephrin family adhesion molecule 1	13.84 (D)	13.31 (D)
Nphs1	Nephrin, NPHS1 adhesion molecule	12.75 (D)	24.07 (D)
Nphs2	Podocin, NPHS2 stomatin family member	4.43 (P)	9.18 (P)
Cd151	CD151 molecule (Raph blood group), CD151 antigen	6.15 (D)	12.25 (D)
Thbd	Thrombomodulin		14.17 (D)
Lmx1b	LIM homeobox transcription factor 1 beta	10.18 (D)	
RAC1	Rac family small GTPase 1		5.86 (D)
SGPL1	sphingosine-1-phosphate lyase 1		17.66 (D)

H

Alternatively Polyadenylated Genes with Novel Roles in Glomerular Disease			
Genes	**Protein**	PAN (%)	ADR (%)
Hsd17b14	hydroxysteroid (17-beta) dehydrogenase 14		45.38 (P)
Dph2	diphthamide biosynthesis 2		41.40 (D)
Dvl3	dishevelled segment polarity protein 3		40.63 (D)
Ccr5	C-C motif chemokine receptor 5		39.56 (D)
Foxn3	forkhead box N3	36.66 (D)	39.54 (D)
Ndrg2	NDRG family member 2		39.28 (D)
Slc25a24	solute carrier family 25 member 24		39.12 (D)
Nup50	nucleoporin 50		39.09 (D)
Cep43	centrosomal protein 43		38.88 (D)
Mgat3	beta-1,4-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase		38.44 (D)

Figure 3

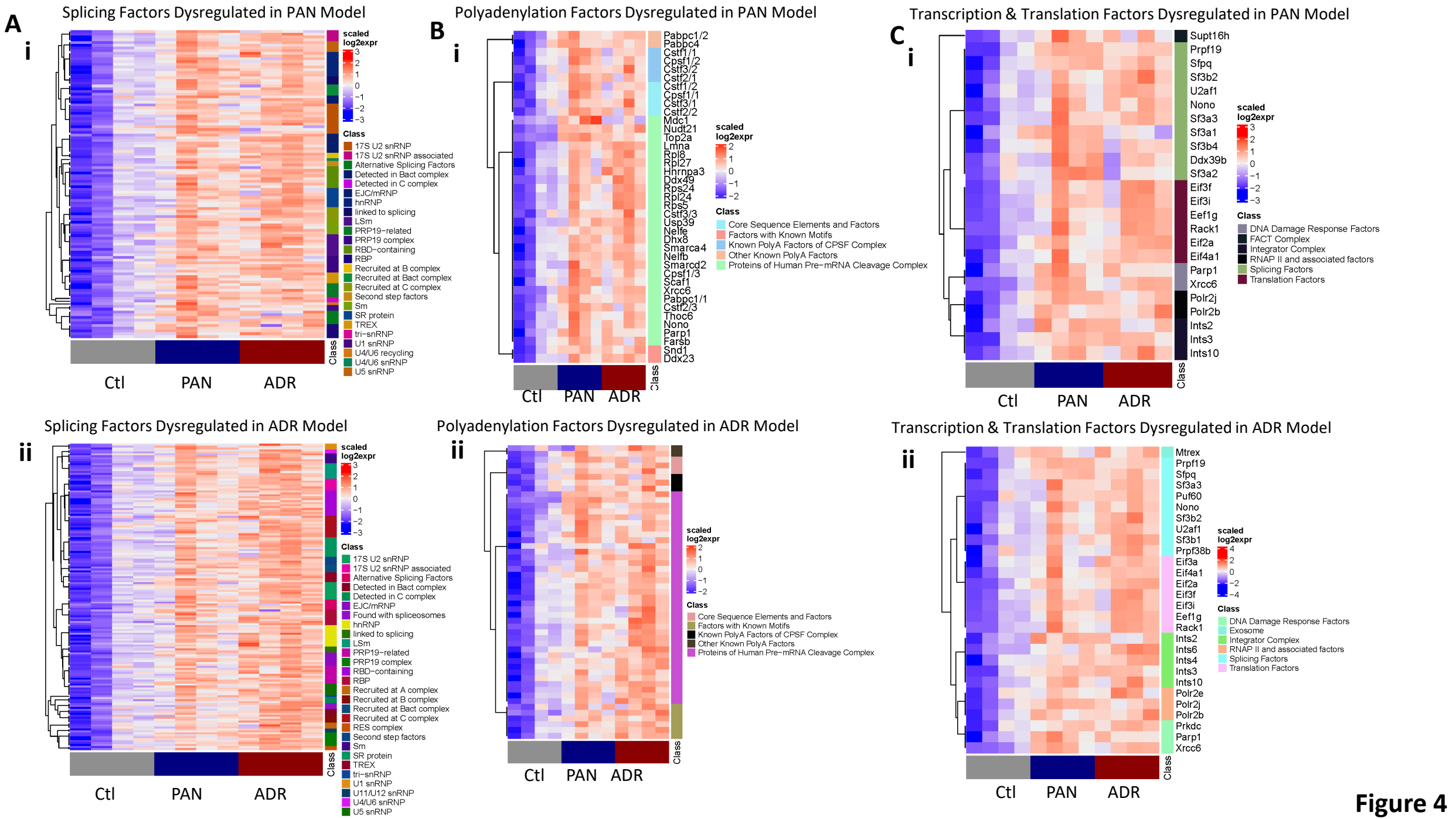

A
i
Splicing Factors Dysregulated in PAN Model
scaled log2expr
3
2
1
0
−1
−2
−3
Class
17S U2 snRNP
17S U2 snRNP associated
Alternative Splicing Factors
Detected in Bact complex
Detected in C complex
EJC/mRNP
hnRNP
linked to splicing
LSm
PRP19−related
PRP19 complex
RBD−containing
RBP
Recruited at B complex
Recruited at Bact complex
Recruited at C complex
Second step factors
Sm
SR protein
TREX
tri−snRNP
U1 snRNP
U4/U6 recycling
U4/U6 snRNP
U5 snRNP
Class
Ctl
PAN
ADR
B
i
Polyadenylation Factors Dysregulated in PAN Model
Pabpc1/2
Pabpc4
Cstf1/1
Cpsf1/2
Cstf3/2
Cstf2/1
Cstf1/2
Cpsf1/1
Cstf3/1
Cstf2/2
Mdc1
Nudt21
Top2a
Lmna
Rpl8
Rpl27
Hnrnpa3
Ddx49
Rps24
Rpl24
Rps5
Cstf3/3
Usp39
Nelfe
Dhx8
Smarca4
Nelfb
Smarcd2
Cpsf1/3
Scaf1
Xrcc6
Pabpc1/1
Cstf2/3
Thoc6
Nono
Parp1
Farsb
Snd1
Ddx23
scaled log2expr
2
1
0
−1
−2
Class
Core Sequence Elements and Factors
Factors with Known Motifs
Known PolyA Factors of CPSF Complex
Other Known PolyA Factors
Proteins of Human Pre−mRNA Cleavage Complex
Class
Ctl
PAN
ADR
C
i
Transcription & Translation Factors Dysregulated in PAN Model
Supt16h
Prpf19
Sfpq
Sf3b2
U2af1
Nono
Sf3a3
Sf3a1
Sf3b4
Ddx39b
Sf3a2
Eif3f
Eif3i
Eef1g
Rack1
Eif2a
Eif4a1
Parp1
Xrcc6
Polr2j
Polr2b
Ints2
Ints3
Ints10
scaled log2expr
3
2
1
0
−1
−2
−3
Class
DNA Damage Response Factors
FACT Complex
Integrator Complex
RNAP II and associated factors
Splicing Factors
Translation Factors
Class
Ctl
PAN
ADR
ii
Splicing Factors Dysregulated in ADR Model
scaled log2expr
3
2
1
0
−1
−2
−3
Class
17S U2 snRNP
17S U2 snRNP associated
Alternative Splicing Factors
Detected in Bact complex
Detected in C complex
EJC/mRNP
Found with spliceosomes
hnRNP
linked to splicing
LSm
PRP19−related
PRP19 complex
RBD−containing
RBP
Recruited at A complex
Recruited at B complex
Recruited at Bact complex
Recruited at C complex
RES complex
Second step factors
Sm
SR protein
TREX
tri−snRNP
U1 snRNP
U11/U12 snRNP
U4/U6 snRNP
U5 snRNP
Class
Ctl
PAN
ADR
ii
Polyadenylation Factors Dysregulated in ADR Model
scaled log2expr
2
1
0
−1
−2
Class
Core Sequence Elements and Factors
Factors with Known Motifs
Known PolyA Factors of CPSF Complex
Other Known PolyA Factors
Proteins of Human Pre−mRNA Cleavage Complex
Class
Ctl
PAN
ADR
ii
Transcription & Translation Factors Dysregulated in ADR Model
Mtrex
Prpf19
Sfpq
Sf3a3
Puf60
Nono
Sf3b2
U2af1
Sf3b1
Prpf38b
Eif3a
Eif4a1
Eif2a
Eif3f
Eif3i
Eef1g
Rack1
Ints2
Ints6
Ints4
Ints3
Ints10
Polr2e
Polr2j
Polr2b
Prkdc
Parp1
Xrcc6
scaled log2expr
4
2
0
−2
−4
Class
DNA Damage Response Factors
Exosome
Integrator Complex
RNAP II and associated factors
Splicing Factors
Translation Factors
Class
Ctl
PAN
ADR

Figure 4

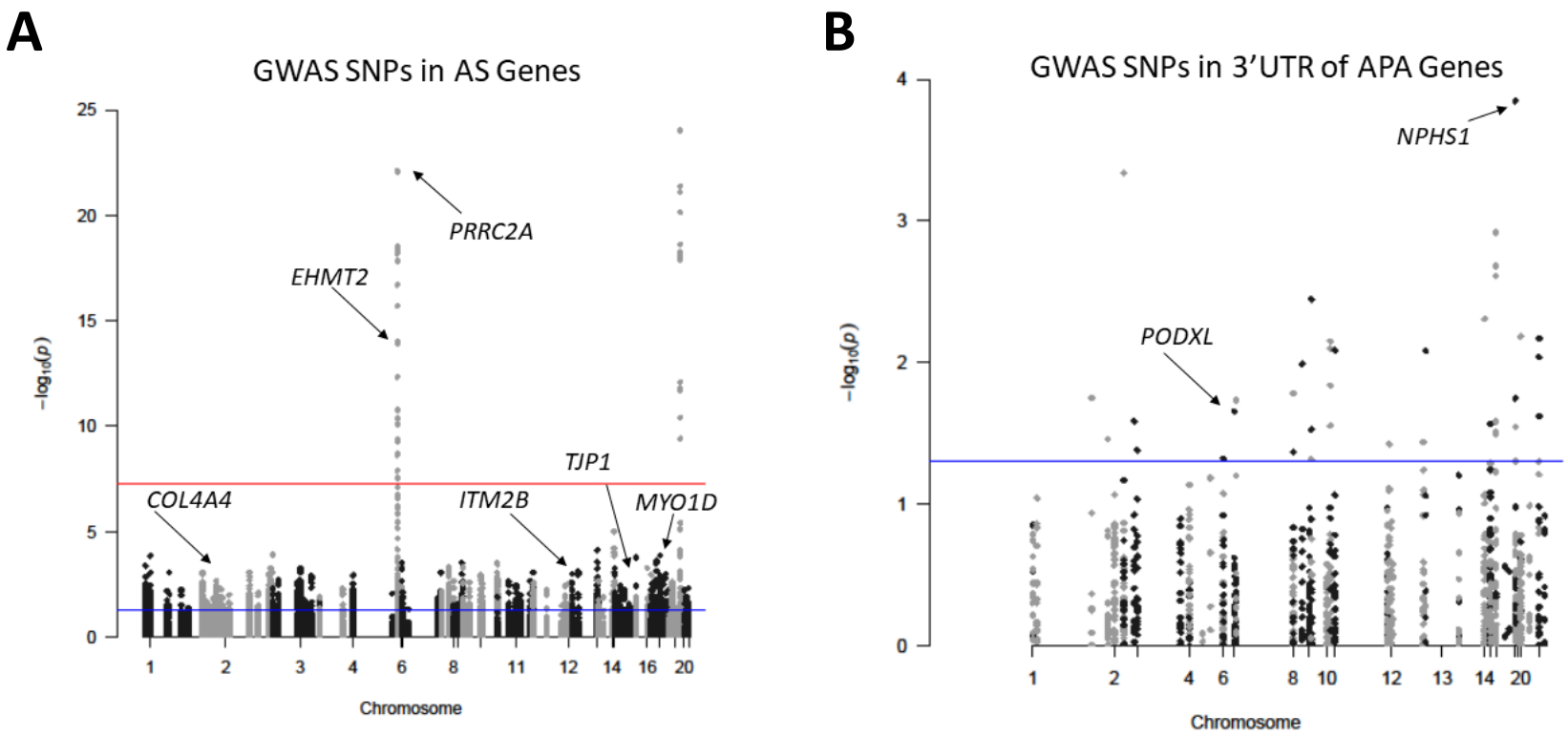

A
GWAS SNPs in AS Genes
PRRC2A
EHMT2
TJP1
COL4A4
ITM2B
MYO1D
−log10(p)
Chromosome
B
GWAS SNPs in 3'UTR of APA Genes
NPHS1
PODXL
−log10(p)
Chromosome

Figure 5