The Role of YY1 in the Modulation of the Podocyte Molecular Phenotype in High Glucose Milieu

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ABSTRACT

APOL1 is a protein implicated in the expression of the molecular phenotype of parietal epithelial cells. Differentiation of parietal epithelial cells into podocytes manifests in the form of downregulation of proliferation markers and upregulation of APOL1. High-glucose induced dedifferentiation of podocytes, as in diabetic nephropathy, causes dedifferentiation of podocytes, manifesting as downregulation of APOL1, upregulation of proliferation markers, and downregulation of critical podocyte proteins that maintain the glomerular filtration barrier. YY1 is a polycomb group protein that is involved in repression of gene transcription, and its function has been implicated previously in the kidney in renal tubular cells, where high YY1 contributed to high epithelial-to-mesenchymal transition that resulted in renal scarring, and low APOL1 was linked to lower renal scarring. In this study, it was hypothesized that YY1 acts to repress APOL1 transcription and preserve the undifferentiated phenotype, while downregulation of YY1 would increase APOL1 expression and yield the differentiated phenotype. The relationship between YY1 and APOL1 was examined through immunoblotting and computational studies. Through immunoblotting studies, it was observed that YY1 and APOL1 are inversely related, and that high glucose conditions resulting in dedifferentiation of podocytes enhance YY1 expression. Computational studies were used to analyze the dynamics of YY1 bound to the polycomb repressor complex. Silencing of YY1 was observed to upregulate APOL1. These findings identify YY1 as a potential therapeutic target for treatment of diabetic nephropathy in the podocyte.

INTRODUCTION

DIABETIC NEPHROPATHY

Diabetic nephropathy (DN), or simply diabetic kidney disease, is a pathological syndrome that arises as a complication of diabetes mellitus that leads to progressive loss of kidney function. Diabetic nephropathy is characterized by damage to the glomerular filtration barrier (which is responsible for filtration of small biomolecules, water, and ionic salts) in the kidney that leads to a universal loss of glomerular filtration rate (GFR); the loss of the glomerular filtration barrier leads to high pathological concentrations of urine albumin in excretion as a result of massive proteinuria, and podocyte injury as a result of high glucose conditions leads to glomerular lesions in the kidney, which are characteristic of glomerulopathies such as focal segmental glomerulosclerosis (FSGS) (Lim 2014).

As of 2014, more than 387 million people have been diagnosed with some form of diabetes, and the figure is projected to reach 592 million in 2035 (Gheith et al. 2015). Diabetic nephropathy occurs in 20% to 40% of all diabetics (although it is more prevalent in type 1 diabetics, where a smaller proportion of type 2 diabetics progress to end-stage renal disease (ESRD)). Cumulatively, diabetic nephropathy is the most frequent cause of ESRD, and diabetic nephropathy patients who reach this point require extensive hemodialysis and, in some cases, kidney transplantation (Aldukhayel 2017). Diabetic nephropathy also increases the 10-year mortality rate amongst diabetics, nearly 6-fold compared to matched non-diabetics. Elucidating the mechanisms by which diabetic nephropathy manifests in the glomerulus, as well as identifying proteins that are involved in maintaining the stability of the podocyte and that are disrupted in diabetic conditions is important to identifying therapeutic targets for treatment purposes.

PODOCYTE, PARIETAL EPITHELIAL CELLS, PODOCYTE MOLECULAR PHENOTYPE

Podocytes are terminally differentiated cells that surround the exterior basement membrane surface of the glomerular capillary (Reiser et al. 2016). They form a major component of the glomerular filtration barrier, which is involved in keeping out plasma proteins from the urinary ultrafiltrate. Podocytes have a highly complex cellular architecture that contains projections from their cell body that extend outwards, wrapping around the glomerular capillary and forming interdigitated foot processes. In aggregate, this dynamic network of foot processes, known as pedicels, forms narrow gaps, or filtration slits, between podocyte pedicels (Pavenstadt 2000). These filtration slits contain cell-surface protein complexes known as slit diaphragms, which cover the filtration slits and participate in podocyte signaling

and restrict the passage of large macromolecules including serum albumin. The filtration slits, however, are permeable to small biomolecules, including glucose, water, and ionic salts; these molecules pass through the filtration slits and form an ultrafiltrate in the renal tubules, where they are exported to the nephron for processing into urine. Contraction and relaxation of these pedicels modulates the glomerular filtration rate which ultimately affects the ultrafiltration coefficient, representative of the volume of fluid that passes through the filtration slits.

Podocyte injury alters the glomerular filtration barrier by causing pedicels to fuse with the membrane, destroying the barrier (Li et al 2008). Early renal dysfunction is characterized by proteinuria which is the massive leak of proteins out of the glomerular structure into the urine (in particular, serum albumin is a major component of this leakage. In the case of diabetic nephropathy, podocytes in high glucose conditions exhibit a phenomenon known as dedifferentiation, characteristic of an attempt to regenerate in adverse conditions. Dedifferentiated podocytes revert back to an undifferentiated phenotype begin to express proteins characteristic of a proliferative state, including PAX2, which is indicative of an attempt to regenerate podocytes in diabetic conditions (Mishra et al 2018).

As previously mentioned, podocytes, whether in healthy or disease phenotypes, are terminally differentiated and are unable to regenerate themselves following injury or destruction. In contrast, parietal epithelial cells retain the ability to proliferate in disease conditions. These cells, attached to the basement membrane of the Bowman's capsule, derive from the same ancestral mesenchymal as podocytes, during which both podocytes and parietal epithelial cells acquire their characteristics during glomerulogenesis (Romagnani 2011). Their exact function has not yet been elucidated, but several studies point to several functions of parietal epithelial cells. Multiple cellular features of parietal epithelial cells are highly consistent with those of tight junctions, which point to parietal epithelial cells serving as a secondary barrier to plasma protein and ultrafiltrate constituent leakage in the Bowman's basement membrane, alongside the glomerular filtration barrier formed by podocyte pedicels (Ohse et al. 2009).

One major function of parietal epithelial cells that is still being explored is that they serve as a reservoir for podocyte regeneration during disease phenotypes. Even under normal, healthy conditions, podocyturia as a result of detachment of podocytes in the urinary space occurs under physiological conditions, and podocytes are lost into the urine. Studies have shown that in physiological conditions in juvenile rats, parietal epithelial cells migrate into the glomerular tuft and differentiate into podocytes. This has been supported by demonstrating the existence of transitional-state cells at the glomerular vascular stalk that exhibit both parietal epithelial cell and podocyte features (Appel et al. 2009), as well as the overlap of molecular phenotype between podocytes and parietal epithelial cells in diabetic nephropathy (Andeen et al. 2015). However, activation of parietal epithelial cells leading to increased proliferation and

migration activity has been shown to be involved in the development of sclerotic lesions and the pathogenesis of multiple glomerular diseases, including focal segmental glomerulosclerosis (FSGS) and rapidly progressing glomerulonephritis (RPGN) (Li et al 2008). In disease states, parietal epithelial cell proliferation and migration activity is linked to the development of crescentic glomerulonephritis, where crescentic lesions are observed (Moeller et al. 2014). While novel treatment methods can arise from elucidating the mechanism by which parietal epithelial cells migrate and differentiate into podocytes at the glomerular tuft, it is important to acknowledge that arbitrary parietal epithelial cell activation might prove detrimental to the efficacy of treatments, and more must be understood if parietal epithelial cell activation is to emerge as a therapy for podocyturia.

APOLIPOPROTEIN L1

The *APOL1* gene encodes apolipoprotein L1 (APOL1), a protein that predominantly binds to HDL particles and circulates through the blood (O'Toole et al. 2017). APOL1 has three forms, a wild-type called G0, and two sequence variants, referred to as G1 and G2. Its secretory function has been ascribed to conferring resistance to trypanosomiasis through lysis of *Trypanosoma brucei* species, as APOL1G0 acts as a trypanolytic molecule when bound to HDL particles (Dummer et al. 2015). However, its exact intracellular role in the podocyte has not yet been described, and is still under active investigation.

Its intracellular expression is highly localized to glomerular podocytes, where APOL1 expression has been correlated with the podocyte molecular phenotype. The APOL1-miR193a axis, a bifunctional relationship between the APOL1 protein and the intracellular microRNA miR193a, has been implicated in the modulation of the podocyte molecular phenotype, especially in high glucose conditions (Kumar et al. 2019). This axis is bifunctional, in that both APOL1 and miR193a regulate each other; high APOL1 leads to low miR193a, and high miR193a leads to low APOL1. High glucose-induced dedifferentiation of podocytes was linked to decreasing APOL1 expression and increasing miR193a expression; it was found that APOL1 expression is critical for maintaining the differentiated podocyte molecular phenotype. One other major role of APOL1 is related to the parietal epithelial cell-podocyte transition as described previously. It has been shown that downregulation of miR193a in parietal epithelial cells induced APOL1 expression, which stimulated the acquisition of a podocyte molecular phenotype, resulting in the transition from being a parietal epithelial cell to being a podocyte (Kumar et al. 2018).

YIN YANG 1 AND POLYCOMB GROUP PROTEINS

Polycomb group (PcG) proteins are a family of epigenetic factors that form highly conserved regulatory complexes that are able to silence gene expression globally (Di Croce et al 2013). Through chemical histone modification, polycomb group proteins are able to remodel chromatin structures and induce gene repression. They are known to be involved in several biological tasks, in particular, proliferation and differentiation.

Of particular focus is Polycomb repressor complex 2 (PRC2), which serves as a source of histone modification for epigenetic gene silencing (Golbabpour et al. 2013). The core catalytic component of PRC2 is composed of enhancer of zeste homolog 2 (EZH2), which endows PRC2 with histone-lysine methyltransferase activity; suppressor of zeste homolog 12 (SUZ12) and embryonic ectoderm development (EED) protein, both of which assist EZH2 in performing histone methylation (and without which EZH2 cannot fully perform its function); and retinoblastoma binding protein 4 (RBBP4), which serves to bind PRC2 to histones for histone modification. The result of PRC2 binding to histones is trimethylation of lysine 27 on histone H3, shortened to H3K27me3, a characteristic of PRC2-induced epigenetic silencing.

Through EED, which is a cofactor of EZH2, PRC2 has been shown to recruit several additional proteins to assist in histone modification and chromatin remodeling, and in particular, PRC2 has been shown to recruit histone deacetylases (HDAC) and DNA methyltransferases (DNMT). Through HDACs, chemical modifications to histones removes any acetyl groups present, which results in aggregation of neighboring nucleosomes and tightens chromatin structure, preventing gene transcription (van der Vlag et al 1999, Junpei et al 2010). Through DNMTs, chemical modifications to chromatin prevent proper binding of transcription factors, which induces gene silencing (Vire et al 2006).

Past studies have implicated the role of PRC2 in the modulation of the podocyte molecular phenotype. In particular, one study (Mishra et al 2018) has shown that formation of a WT1 repressor complex containing PRC2 components that binds to the promoter of PAX2, a transcription factor that is involved in cellular proliferation. Ectopic expression of PAX2 has been demonstrated in podocytes in diabetic conditions, where dedifferentiation of differentiated podocytes led to increased expression of PAX2 and lower expression of WT1 and the repressor complex components. Downregulation of PAX2 is a characteristic of podocyte differentiation, as it is expressed in parietal epithelial cells and undifferentiated podocytes, and is seldom expressed in differentiated podocytes.

However, on its own, PRC2 does not intrinsically have the capability to bind to chromatin and perform chromatin remodelling or histone modification (Laugesen et al 2019). In the case of WT1, as previously mentioned, it has been inferred that WT1 serves as a recruitment platform for PRC2 components, whereby WT1 binds to consensus sequences on the PAX2 promoter and recruits PRC2 to

that position for chromatin remodelling through chemical modification. No previous literature has shown that WT1 or any other transcription factor binds to the APOL1 gene for gene silencing.

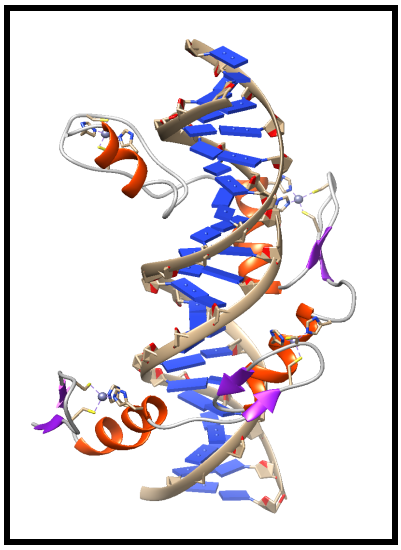


Figure 1: Model of zinc finger regions of YY1 bound to a DNA sequence (PDB ID: 1ubd). Zinc finger alpha helices are highlighted in orange. Image was generated using UCSF Chimera.

YY1 is a transcription factor belonging to the GLI-Kruppel class of zinc-finger proteins. It exhibits positive and negative control on a large number of cellular genes by binding to sites overlapping the transcriptional start site (Gordon et al 2006). In particular, through a domain on YY1 known as the REPO domain (amino acids 201-226), YY1 has been shown to recruit polycomb group proteins, specifically those from PRC2, to DNA sequences for chromatin remodelling (Wilkinson et al 2006). In addition, YY1 has been shown to be involved in the epithelial-to-mesenchymal transition of renal tubular cells in high glucose conditions, where YY1 expression was upregulated in a glucose-and-time dependent manner, and where upregulation of YY1 promoted renal fibrosis of renal tubular cells through inducing epithelial-to-mesenchymal transition, whereas downregulation of YY1 reversed renal fibrosis by reducing this epithelial-to-mesenchymal transition (Yang et al 2019).

HYPOTHESIS AND PURPOSE

Since polycomb group proteins have been implicated in the maintenance of the podocyte molecular phenotype, and that YY1 both recruits polycomb group proteins and has been shown to play a role in diabetic nephropathy (specifically, high glucose-induced renal fibrosis of renal tubular cells), the role of YY1 in the modulation of the podocyte molecular phenotype in relation to APOL1 was explored in this study. Specifically, the relationship between APOL1 and YY1, and how YY1 interacts with

APOL1 in the cell, was examined. In addition, molecular dynamics studies were used to examine the internal dynamics of the PRC2 complex containing YY1 and either DNMT1 or HDAC1. Examining how YY1 interacts with APOL1 would provide more insight into the role of APOL1 in the podocyte, as well as potentially identifying new therapeutic targets that could be used to induce APOL1 expression in parietal epithelial cells and artificially cause differentiation of podocytes to repair the glomerular filtration barrier in high glucose conditions.

METHODS

SEQUENCE RETRIEVAL, AB INITIO MODELLING, MOLECULAR DOCKING

The sequences for the polycomb proteins YY1, EZH2, EED, SUZ12, RBBP4, and DNMT1 and HDAC1 were obtained from the Uniprot database. To model each protein, the ITASSER protein modelling server was used. Following modeling, the cryo-EM structure of PRC2 was retrieved from the EMDataBank (EMD-7334), and the flexible-body docking software MVP-Fit was used to perform flexible-body fitting and docking of EZH2, EED, SUZ12, and RBBP4 into the cryo-EM density map.

The generated YY1 model was docked to a DNA sequence representing a predicted YY1 binding site in the APOL1 promoter using NPDock. The YY1 model was docked to the PRC2 model generated from cryo-EM docking using the ClusPro server, and the structure with the greatest cluster size that favored van der Waals and electrostatic interactions was chosen. DNMT1 and HDAC1 were docked to this YY1-PRC2 complex through ClusPro as well, and the structure with the greatest cluster size that favored van der Waals and electrostatic interactions was chosen. Using PyMOL, the structure of YY1 bound to DNA and the PRC2-DNMT1 and PRC2-HDAC1 containing YY1 were aligned, and the structures of YY1-PRC2-DNMT1 and YY1-PRC2-HDAC1 were generated.

MOLECULAR DYNAMICS SIMULATIONS

Following cryo-EM docking, molecular evolution of protein-DNA and protein-ligand complexes were studied through molecular dynamics simulations. Simulations of the YY1-DNA-PRC2 complexes were carried out using the GROMACS 2019.2 molecular dynamics software package using the Martini force field. Simulations were carried out on a Dell XPS Desktop with an RTX 2070 graphics card to enable GPU acceleration of GROMACS.

Due to the size of the YY1-DNA-PRC2 complexes, these complexes were subjected to coarse-grained molecular dynamics, which reduces the degrees of freedom by grouping atoms together, increasing computational efficiency at the cost of atomic-level accuracy. Using the Martini coarse-grained

force field, all YY1-DNA-PRC2 complexes were converted to their CG representations using the *martinize* and *martinize-dna* Python scripts. Following this, all complexes were subjected to a short 100 step energy minimization *in vacuo*, to remove any initial steric clashes and poor contacts in the initial structure. The complexes were then solvated in an explicitly represented cubic Martini water box extending 2.5 nm from the center of mass of the complexes in all directions. To neutralize charges and produce a physiological salt concentration of 150 mM, water molecules were replaced by Na+ and Cl- ions. The solvated and ionized complexes were then relaxed by 5000 steps of energy minimization to remove any further steric clashes or poor contacts in the complexes and the solvent. Following energy minimization, a 1 ns NVT equilibration (constant number of particles, volume and temperature) was performed, with position restraints on all backbone atoms of the complexes, to allow the solvent to equilibrate to 325K. Afterwards, another 1 ns NVT equilibration was performed, this time without position restraints, to allow the complexes to reach 325K and equilibrate within the solvent. 325K was chosen as the starting temperature for the simulation to accelerate any protein dynamics or structural changes during the simulation.

Following equilibration, each system underwent a 250 ns production run with a 20 fs integrator timestep. The system temperature was coupled to the protein-DNA and solvent-ion groups, respectively, using the V-rescale modified Berendsen thermostat to maintain the temperature at 325K. The system pressure was coupled to the same groups as before, using the Parrinello-Rahman pressure coupling method to maintain the pressure at 1 bar. Trajectories from the simulations were recorded every 1 ns.

Analysis of CG-MD trajectories was performed using the *gmx rms, gmx rmsf,* and *gmx gyrate* distribution programs in GROMACS. Data was plotted using XMGrace.

HUMAN PODOCYTE

Human podocytes were immortalized by transfection of a temperature-sensitive simian virus 40T antigen. This enabled the cells to proliferate at a growth-permissive temperature (33°C) and enter growth arrest at a non-growth-permissive temperature (37°C). The growth medium used to culture the cells contained RPMI 1640, which was supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 1X penicillin-streptomycin, and 1X insulin-transferrin-selenium (Invitrogen). Undifferentiated podocytes were seeded on plates coated with collagen, and were allowed to differentiate through preincubation in normal RPMI 1640 containing 10 mM glucose for 10 days at 37°C. Preceding experimentation, differentiated podocytes were washed three times with glucose and serum-free media. During experimentation, differentiated podocytes were incubated in glucose and ITS-free RPMI containing either no glucose or 30 mM of glucose (defined as a high glucose concentration) for 48 hours.

SILENCING OF YY1

To silence specific proteins of interest, differentiated podocytes were transfected with scrambling small interfering RNA (SCR) as a negative control or 25 nM YY1 siRNA (Santa Cruz Biotechnology) with Lipofectamine RNAiMAX transfection reagent according to the manufacturer's protocol (Thermofisher Scientific). 9 uL of Lipofectamine reagent and 3 uL of 10 mL siRNA were diluted in 150 uL of Opti-MEM (ThermoFisher Scientific). 150 uL of diluted siRNA was added to 150 uL of Lipofectamine reagent, and the mixture was incubated for 5 minutes at 25C (room temperature). Differentiated podocytes at 60-80% confluence were transfected in 6-well plates by the addition of the siRNA-lipid complex, and kept at 37C in Opti-MEM for 48 hours. The cells were harvested for protein analysis through SDS-PAGE and Western blotting.

WESTERN BLOTTING STUDIES

As previously described, Western blotting studies were conducted to determine relative protein concentrations across cell lysates. Control and experimental cells were lysed using RIPA buffer containing 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% deoxycholate, 0.1% SDS, 1X protease inhibitor cocktail set (Calbiochem), 1 mM PMSF, and 0.2 mM sodium orthovanadate. Protein concentrations of cell lysates were measured using a Bio-Rad protein assay kit. A total of 30 ug of protein lysate extract was loaded into each gel lane. Samples were loaded onto a 10% (acrylamide concentration) sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes processed for immunoblotting with primary antibodies (1/1000 dilution) against APOL1, YY1, nephrin, podocalyxin, and CD2AP and their respective controls, followed by treatment with the corresponding horseradish peroxidase-labeled secondary antibodies. Blots were developed using a Bio-Rad chemiluminescence detection machine. To confirm equal protein loading and successful protein transfer, immunoblotting for determination of GAPDH protein using the respective polyclonal antibodies (Santa Cruz Biotechnology) on the same Western blots.

RESULTS AND DISCUSSION

HIGH GLUCOSE-INDUCED DEDIFFERENTIATION UPREGULATES YY1 AND DOWNREGULATES APOL1

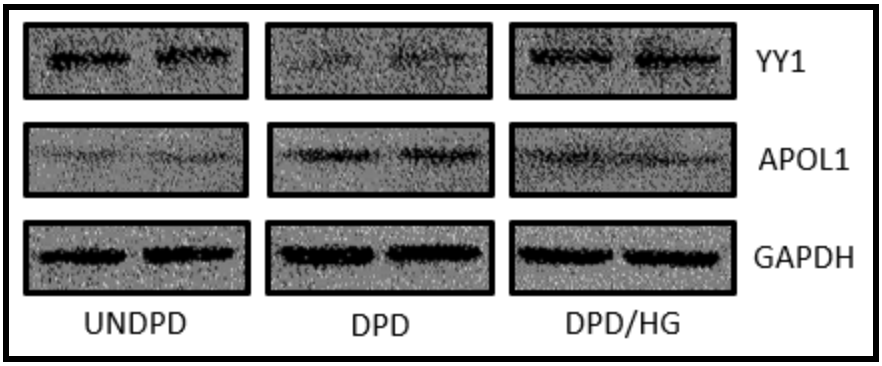


Figure 2: Cellular lysates of UNDPDs, DPDs, and DPDs treated with high glucose (incubated in 30 mM glucose in RPMI 1640 for 48 hours) were probed for APOL1 and YY1. GAPDH was probed as a control (n=2).

Cellular fractions of undifferentiated podocytes, differentiated podocytes, and differentiated podocytes incubated in high glucose media were probed for YY1 and APOL1, and reprobed for GAPDH as a control. In undifferentiated podocytes, YY1 is expressed, whereas APOL1 shows very low levels of expression. Differentiated podocytes display attenuated expression of YY1, and comparatively increased expression of APOL1. Treatment of differentiated podocytes with high glucose media resulted in an increase in YY1 expression, and a decrease in APOL1 expression. This particular experiment demonstrates the expressional relationship between APOL1 and YY1, and establishes a basis for their connection in the context of the podocyte and diabetic nephropathy. Since YY1 is a transcription factor, its role is heavily involved in modulating the expression of specific genes by binding to those genes. It is plausible that YY1 affects APOL1 in some way, or that the relationship is purely expressional (such that modulation of YY1 expression does not actually affect the expression of APOL1).

YY1 SILENCING IN UNDIFFERENTIATED PODOCYTES INCREASES APOL1 EXPRESSION

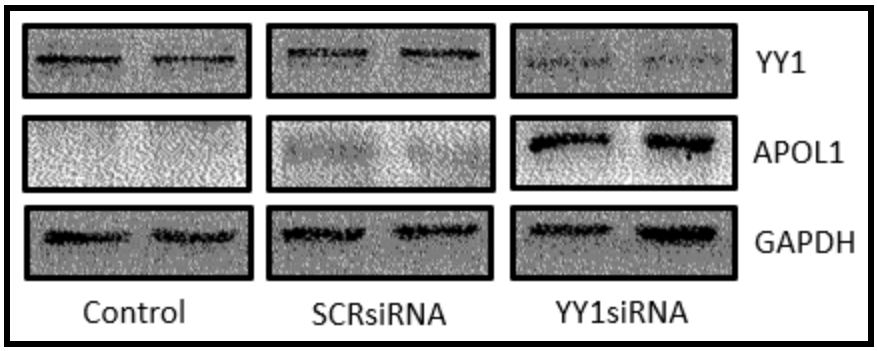


Figure 3: UNDPDs were transfected with either scrambling or YY1siRNA using Lipofectamine RNAiMAX, and incubated in Opti-MEM for 48 hours. Cellular lysates of UNDPDs were probed for YY1 and APOL1. GAPDH was probed as a control (n=2).

Undifferentiated podocytes were treated with scrambled (SCR) and YY1 siRNA, and cellular fractions were probed for APOL1 and YY1, and reprobed for GAPDH as a control. Treatment with YY1 siRNA, compared to control lysates, was observed to result in upregulation of APOL1 expression. This experiment highlights that modulating YY1 expression affects the expression of APOL1, indicating that YY1 affects APOL1 expression through some mechanism. Whether the relationship is direct (YY1 directly targets APOL1) or indirect (YY1 targets other proteins that ultimately affect APOL1 expression) is unclear.

How directly modulating YY1 expression might affect podocyte integrity must be considered if YY1 is to be considered a potential therapeutic target. Knockdown of YY1 has been observed to ameliorate high glucose-induced renal fibrosis in renal tubular cells in diabetic nephropathy conditions (Yang et al. 2019). It was shown that overexpression of YY1 induced renal fibrosis in mice through upregulation of a-SMA expression, whereas downregulation of YY1 was able to reverse high glucose-induced renal fibrosis through improvements in the epithelial-to-mesenchymal transition of renal tubular cells, both *in vivo* and *in vitro*. Since increased expression of wild-type APOL1 is linked to the podocyte phenotype, and since low YY1 expression is correlated with high APOL1 expression, it is possible that inhibition of YY1 might be used to increase expression of APOL1, which would induce the podocyte molecular phenotype through downregulation of PAX2 and upregulation of WT1 (both of which are expressionally, but not mechanistically, related, and characterize the podocyte molecular phenotype). However, since overt expression of APOL1 risk variants is linked to an increased risk of kidney disease phenotypes, it can be inferred that inhibition of YY1 *in vivo* might result in increased prevalence of kidney disease phenotypes and markers. Therefore, although it can be inferred that YY1 could be used as a therapeutic target for causing podocyte differentiation, it must be noted that APOL1 risk variants might prevent the treatment from working as intended.

YY1 SILENCING UPREGULATES EXPRESSION OF SLIT DIAPHRAGM PROTEINS

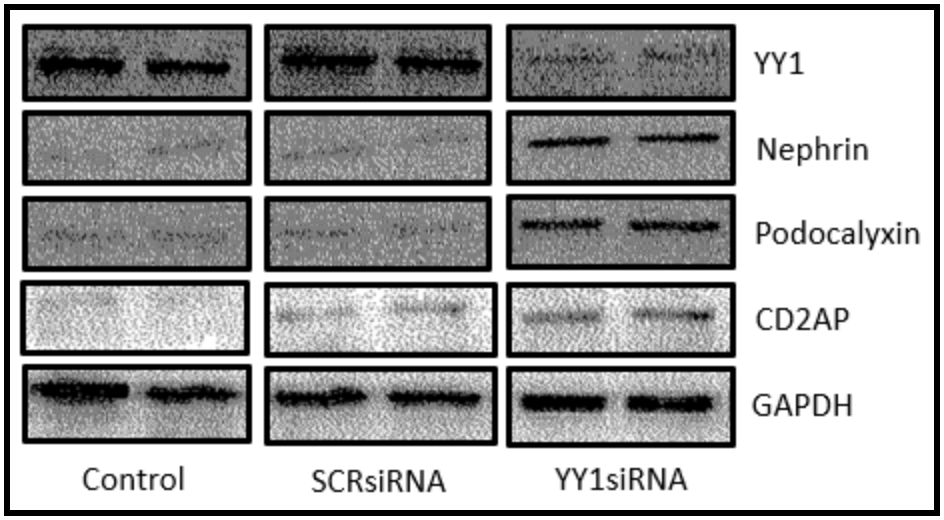


Figure 4: UNDPDs were transfected with either scrambling or YY1siRNA using Lipofectamine RNAiMAX, and incubated in Opti-MEM for 48 hours. Lysates of UNDPDs were probed for YY1, APOL1, nephrin, podocalyxin, and CD2AP. GAPDH was probed for as a control (n=2).

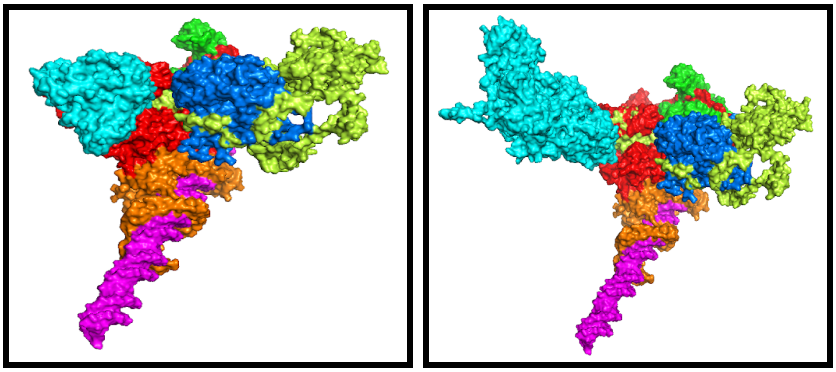
Undifferentiated podocytes were treated with scrambled (SCR) and YY1 siRNA, and cellular fractions were probed for YY1, nephrin, podocalyxin, CD2AP, and reprobed for GAPDH as a control. Treatment with YY1 siRNA, compared to control lysates, was observed to result in upregulation of nephrin, podocalyxin, and CD2AP, three proteins that are a part of the slit diaphragm protein complexes that enable podocytes to properly filter out blood in the glomerulus.

In the previous experiment, it was observed that silencing of YY1 caused an upregulation of APOL1 expression. Since APOL1 expression is directly correlated with the podocyte molecular phenotype, all together, silencing of YY1 increases the expression of APOL1 and slit diaphragm proteins, all of which are characteristic of the molecular phenotype of the podocyte. This indicates that silencing of YY1 may be a potential way to promote the expression of the podocyte molecular phenotype. Since YY1 is highly expressed in undifferentiated podocytes, which are analogous to parietal epithelial cells in their molecular phenotype, it is possible, then, that silencing YY1 in parietal epithelial cells may be a viable way to induce the upregulation of APOL1 and other proteins characteristic of podocytes, serving as a way to artificially generate podocytes during disease conditions, which could allow people with diabetic nephropathy to have their kidney function potentially restored over time.

STRUCTURE OF YY1-PRC2-HDAC1 AND YY1-PRC2-DNMT1

In order to conduct molecular dynamics analysis of the PRC2 containing YY1, HDAC1 or DNMT1, all bound to a YY1 consensus sequence, the structure of YY1 containing PRC2 and

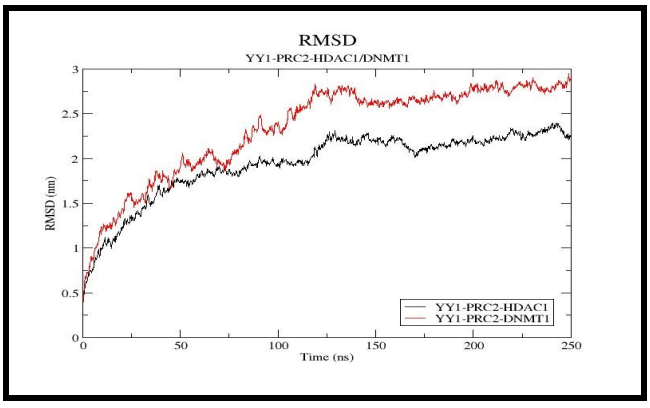
HDAC1/DNMT1 was needed. Through flexible-body docking and fitting of protein structures generated using the ITASSER server, as well as protein-protein docking using Cluspro and protein-DNA docking using NPDock, the structures of YY1-PRC2-HDAC1 and YY1-PRC2-DNMT1 were determined. Surface representations of the complexes are pictured below.

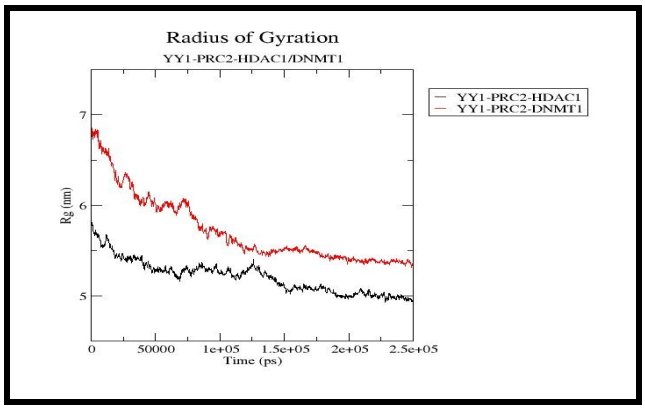


Figures 5 and 6: Structures of YY1-PRC2-HDAC1 (left) and YY1-PRC2-DNMT1 (right) after cryo-EM docking. YY1 is colored in orange, HDAC1 and DNMT1 are colored in cyan. Images were generated using Pymol.

MOLECULAR DYNAMICS ANALYSIS OF YY1-CONTAINING PRC2 BOUND TO DNA IN A COARSE GRAINED MODEL

The RMSDs of the protein-DNA complexes from equilibrated coordinates were evaluated to determine the structural stability of the complex over time, as well as evaluate any deviations in structure that might provide insight into conformational changes. Both complexes reach a stable equilibrium around 125 ns, without any major deviations that would indicate structural instability. In addition, both the YY1-PRC2-HDAC1 and YY1-PRC2-DNMT1 follow a very similar pattern of RMSD from equilibrated coordinates, which indicates that the overall structures of the complexes follow a rather similar evolution. This alignment of deviation from initial structures between the two complexes highlights that the internal dynamics of the systems may follow a very similar mechanism, so it is possible that the mechanism by which YY1-PRC2 in complex with either HDAC1 or DNMT1 exerts its repressive action on genes.





Figures 7 and 8: RMSD and radii of gyration averaged from 250 ns trajectories of PRC2 bound to YY1 on the APOL1 promoter, containing either HDAC1 (YY1-PRC2-HDAC1) or DNMT1 (YY1-PRC2-DNMT1). RMSDs and RGs were measured from starting (equilibrated) coordinates.

The shifts in RMSD, at around 125 ns and 150 ns, indicate that there might be conformational changes in the protein complexes. The radius of gyration gives some insight into what that conformational change might be, since a constantly decreasing radius of gyration is observed. Since radius of gyration is representative of the size of the complex from its center to its outermost point, it can be inferred that as the system evolves, the protein complex collapses inwards, with the most terminal groups (in this case, HDAC1 and DNMT1) folding into the structure, which would decrease the radius of gyration of the protein complex. This may lend some insight into how proteins like HDAC1 and DNMT1 might function when bound to the PRC2, as they might bend from protruding away from the DNA strand to coming closer to the strand, which might allow the protein to exert its effect. In the case of DNMT1, that effect would be DNA methylation. In the case of HDAC1, that would be histone deacetylation, so future computational studies should explore how the histone proteins interact with PRC2 to determine any conformational changes or interactions between the two.

In addition, to analyze the behavior of YY1 in the complexes and identify flexible regions of YY1, per-residue root mean square fluctuation (RMSF) analysis was conducted on each complex based on starting coordinates.

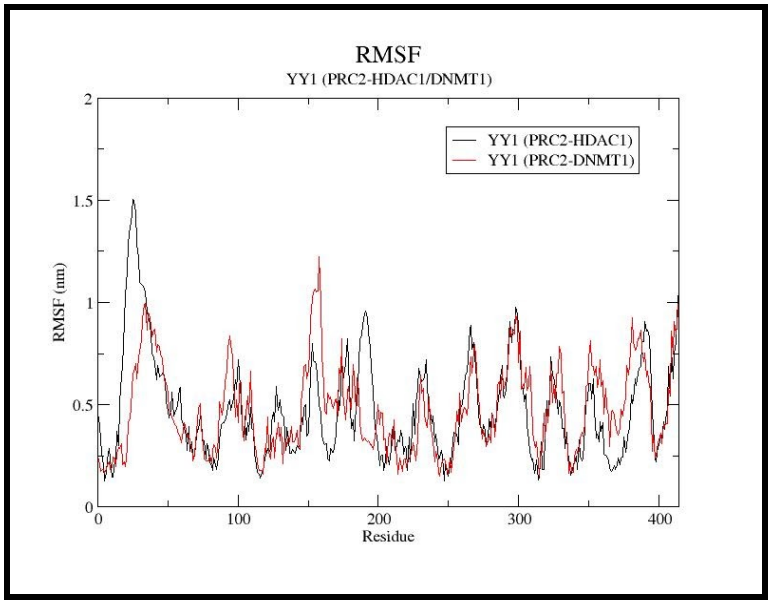


Figure 9: RMSF of YY1 residues 1-414 averaged over the 250 ns trajectories from the PRC2-HDAC1 and PRC2-DNMT1 complexes. RMSF was calculated from initial (equilibrated) coordinates at the start of the production run.

The presence of many peaks in the RMSF graphs for both structures is due to the presence of many highly disorganized loop regions of the YY1 model which lack any significant secondary structure; RMSF peaks in the residue region from 1-295 are insignificant in the context of YY1-PRC2-HDAC1 and YY1-PRC2-DNMT1. The zinc finger regions of YY1 (296-320, 325-347, 353-377, 383-407) are critical to the function of YY1, and so the fluctuation of these regions was chosen for analysis. Four distinct peaks are observed in these regions, which indicates that these zinc finger regions exhibit large fluctuations during the simulation. In addition, the per-residue fluctuations for YY1 align very closely between the PRC2 containing HDAC1 and the PRC2 containing DNMT1, which indicates that YY1 behaves very similarly between the two complexes when bound to the PRC2. It can be inferred that HDAC1 and DNMT1 are both equally as stable with respect to complex formation, although more in-depth analyses (like thermodynamic calculations), including free-energy calculations) might be needed to analyze this in-depth. Such calculations might give us more insight into the prevalence of the formation of one complex over another, which could guide future experimental studies exploring the PRC2.

LIMITATIONS AND FUTURE DIRECTIONS

In this study, the relationship between YY1 and APOL1 expression was evaluated in the undifferentiated, differentiated, and dedifferentiated states. The data has shown that APOL1 and YY1 are inversely regulated, and YY1 is overtly expressed in undifferentiated podocytes and minimally expressed in differentiated podocytes. Knockdown of YY1 was shown to result in increased APOL1 expression, along with other podocyte markers. Treatment with high glucose was shown to upregulate YY1 expression and attenuate APOL1 expression. The data suggests that APOL1 is regulated by YY1, but whether this relationship is directly affecting APOL1 or through other podocyte proteins is unclear and needs to be explored.

Since YY1 is suggested to regulate APOL1, YY1 may serve as a target for therapeutic treatment in diabetic nephropathy, where knockout of YY1 could induce upregulated APOL1 expression, resulting in differentiation of podocytes to replenish those that have undergone dedifferentiation or cell death due to high glucose conditions. However, the data does not explicitly demonstrate this relationship. Since YY1 is a transcription factor, its regulatory properties emerge through binding to regions upstream of transcriptional start sites for chromatin remodelling. Although this study has demonstrated the relationship between APOL1 and YY1, direct binding of YY1 to the APOL1 gene was not examined. Further research should examine the binding of YY1 to the APOL1 gene or examine other podocyte

proteins through which YY1 may exert its effect, through chromatin immunoprecipitation of the YY1 protein and immunoblotting of the precipitated complex to examine the constituents of the YY1 complex.

The study has also made use of undifferentiated podocytes instead of parietal epithelial cells to make conclusions about the role of YY1 in modulation of the podocyte molecular phenotype. Previous studies have examined the relationship between the podocyte and the parietal epithelial cell, where parietal epithelial cells, in disease phenotypes, were shown to have migratory capabilities and migrated to the glomerular tuft for differentiation into podocytes *in vivo*. Although parietal epithelial cells and podocytes are molecular analogs with respect to the proteins of interest *in vitro*, they differ in their location in the glomerulus and Bowman's capsule, and parietal epithelial cells exhibit expression of proteins that contribute to parietal epithelial cell-specific phenotypes, including tight junction formation. Further research could examine the role of YY1 in the parietal epithelial cell *in vitro*; however, future directions should include examining YY1 expression *in vivo* to see how modulation of YY1 expression *in vivo* might directly impact the demonstrated ability of parietal epithelial cells to migrate and differentiate into podocytes.

With respect to computational studies, this study has evaluated the dynamic evolution of YY1-containing PRC2 bound to a YY1 consensus sequence from the APOL1 promoter region in coarse-grained detail. Coarse-grained studies offer increased computational efficiency due to the grouping of atoms to reduce the degrees of freedom of a given system of interest, at the cost of molecular accuracy. Although the YY1-containing PRC2 and its molecular evolution over time has been examined, future directions could include analyzing the complex in fully atomistic detail to analyze how YY1 interacts with DNA and how its DNA-binding properties are modulated when bound to the PRC2 complex. In addition, the study examined the evolution of YY1-PRC2 over a timeframe of 250 ns. Although this time frame is lengthy, the time scale of many of these molecular events is much longer, and so future research could include examining the evolution of YY1-PRC2 over longer time scales, specifically reaching into the micro-and-millisecond range. Also, more in-depth analyses, including analysis that takes into account major biological motions including covariance and eigenvector analysis, and thermodynamics analysis to see the energetic stabilities of the complexes, should be used to analyze complex formation.

CONCLUSION

In this study, it has been demonstrated that in the podocyte, YY1 is inversely related to APOL1, where high YY1 is correlated with low APOL1 in undifferentiated podocytes, and vice versa in differentiated podocytes. Silencing of YY1 expression increased APOL1 expression, indicating that YY1

regulates APOL1 expression in some way. Coarse-grained molecular dynamics simulations were also utilized to examine the binding of YY1 and PRC2 proteins to a YY1 binding site on the APOL1 promoter over a 250 ns timeframe, which gives us insight into the internal dynamics of YY1 and PRC2 proteins bound to DNA. These findings indicate that YY1 might be a future target for therapeutic treatment of diabetic nephropathy by inducing differentiation of parietal epithelial cells into podocytes, but further research is needed to fully implicate YY1 in the modulation of the podocyte molecular phenotype and evaluate how YY1 modulation affects the podocyte both *in vitro* and *in vivo*.

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