Uncovering the Podocyte Foot Process Proteome

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Abstract

Kidneys are a vital organ responsible for filtering and removing toxins from the blood. The major filtration unit of the kidney is the nephron. The nephron consists of the glomerulus and tubules. The glomerulus consists of three components: fenestrated endothelium, the glomerular basement membrane (GBM), and podocytes. Podocytes are highly specialized epithelial cells with long protrusions called foot processes that connect to each other via specialized junctions called slit diaphragms (SD). The SD is largely responsible for the integrity of the glomerulus. Lack of the SD contributes to a loss of podocyte integrity; however, only a small number of proteins are known to localize in the SD. One protein known to localize in the SD is podocin. We utilized proximity dependent Biotin Identification (BioID). We utilized CRISPR-Cas9 based gene editing to knock-in the BioID moiety to the endogenous NPHS2 locus (Podocin). BioID allows for biotinylation of a protein of interest (POI) by fusing a mutated promiscuous prokaryotic biotin ligase to the endogenous locus of our target gene, NPHS2 (Podocin). This biochemical assay allowed us to identify the repertoire of proteins within the podocyte foot process. We identified a novel Immunoglobulin-like domain-containing receptor 2 (Ildr2) within the podocyte SD. Recent data from sc-RNAseq databases evince Ildr2 expression in human podocytes. By utilizing zebrafish and mice, the importance of this novel immunoglobulin family protein can be tested in podocyte integrity. Our innovative method has allowed us to identify novel proteins of the SD and potential new therapeutic targets for kidney disease.

Hypothesis

We hypothesize yet unidentified components of the slit diaphragm are integral for podocyte integrity and kidney function.

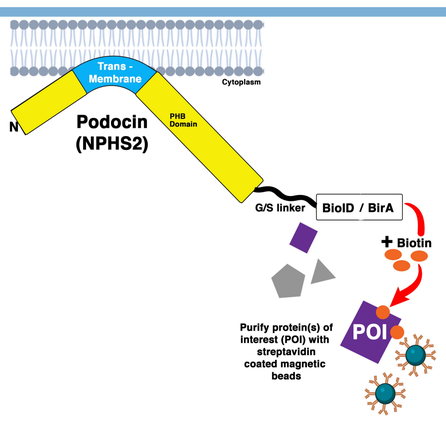


Figure 1. Schematic of protein isolation method, BioID and its components NPHS2 gene product, podocin, was used as a handle to identify the podocyte foot process and slit diaphragm proteome by using a new method of protein isolation, proximity dependent Biotin Identification (BioID). This method of protein isolation utilizes a mutated promiscuous prokaryotic biotin ligase (BirA), alongside a 13 x G / S linker, and an HA tag in order to label proteins of interest neighboring podocin. Biotin is injected into mice with this CRISPR-Cas9 mediated knock-in, and biotinylated neighboring proteins are captured using streptavidin coated magnetic beads.

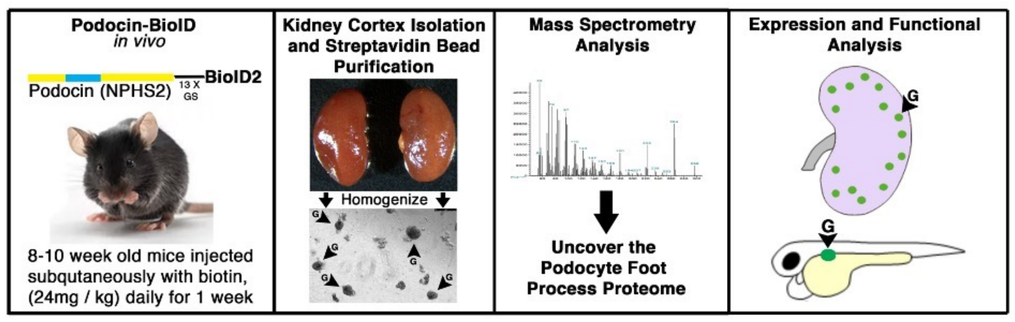


Figure 2. Schematic of how biotinylated proteins are identified using BioID The Podocin-BioID knock-in was injected with biotin every day for one week. The kidney cortex was isolated and homogenized and sent for mass spectrometry. After this, further analysis of expression was performed via *in situ* hybridization and immunofluorescence. This innovative method of protein isolation allows us to identify key proteins of the podocyte foot process and slit diaphragm.

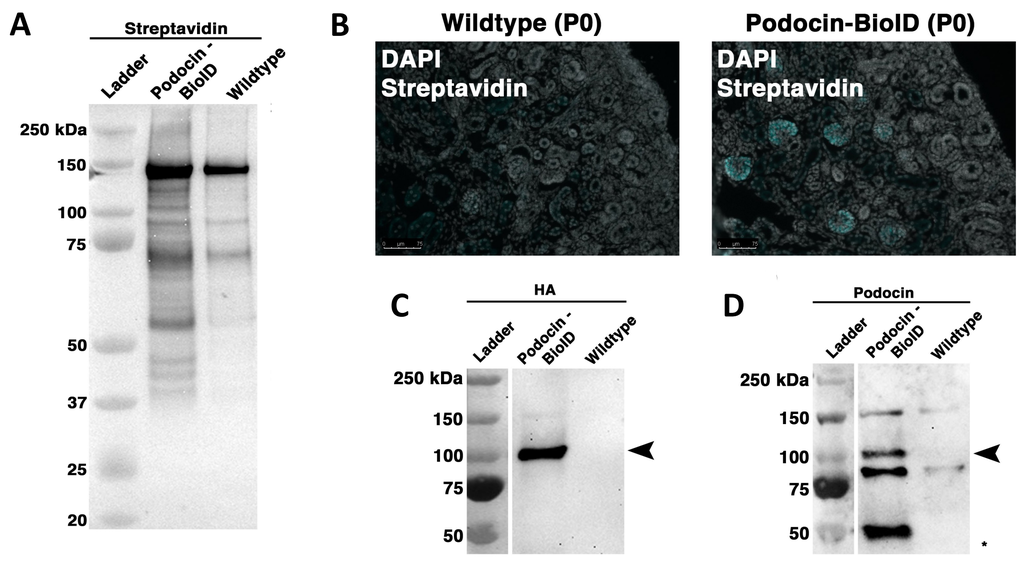


Figure 3. Functional validation of Tg(Podocin-BioID After injecting biotin subcutaneously, mouse kidneys are removed and the cortex surgically isolated to enrich for glomeruli. An aliquot of protein lysate was removed and blotted for Streptavidin. **(A)** A significant enrichment of biotinylated proteins was observed in Podocin-BioID as compared to wildtype control littermates. **(B)** Pregnant dames were injected with biotin every day for one week. IF analysis of the newborn kidneys evinced an enrichment of streptavidin specifically in the glomeruli of Tg(Podocin-BioID) pups (white arrowheads) yet not in control littermates. Our Podocin-BioID mice contain an HA tag within the BioID2 moiety. **(C)** The HA tag is evident only in Podocin-BioID lysates at approximately 100 kDa (arrowhead). **(D)** Probing our Podocin-BioID heterozygous and wildtype lysate samples for Podocin we observe two specific bands in the Podocin-BioID sample at approximately 100 kDa for the HA tagged Podocin-BioID (arrowhead) and a second at approximately 50 kDa for the endogenous unmodified Podocin protein marked with an asterisk (*).

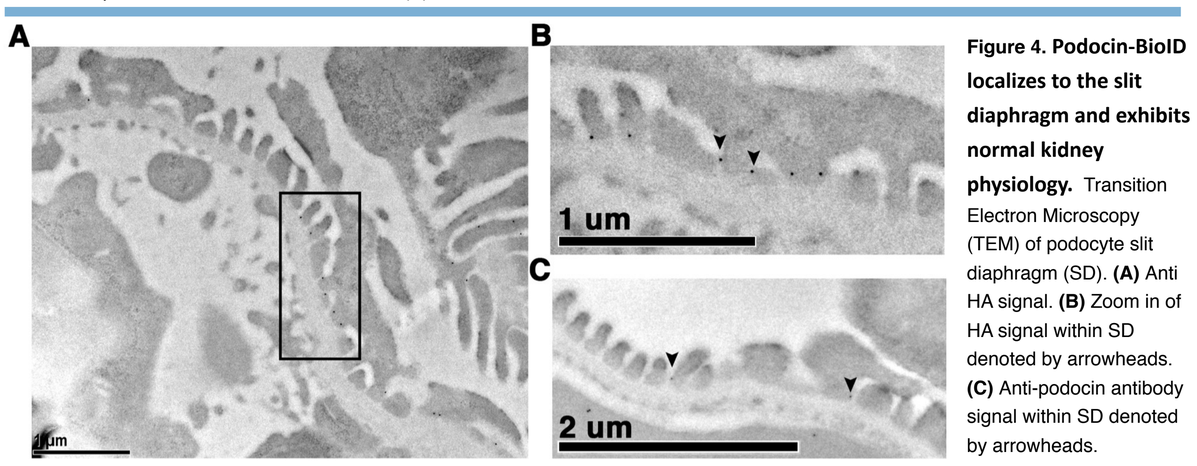
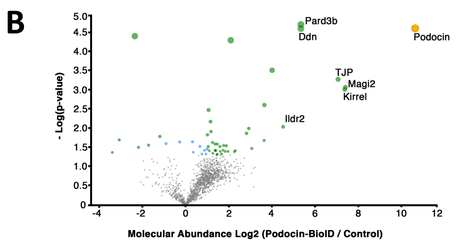


Figure 4. Podocin-BioID localizes to the slit diaphragm and exhibits normal kidney physiology. Transition Electron Microscopy (TEM) of podocyte slit diaphragm (SD). **(A)** Anti HA signal. **(B)** Zoom in of HA signal within SD denoted by arrowheads. **(C)** Anti-podocin antibody signal within SD denoted by arrowheads.

A

Protein Name	Gene Name	Log2 Fold Change	Student's t-test, p-values
Podocin	Nphs2	10.63052265	0.00003
Tight junction protein ZO-1 / 2	Tjp1 / 2	7.398917951	0.0009
Membrane Associated Guanylate Kinase, WW and PDZ Domain Containing 2	Magi2	7.367575643	0.001
Kin of IRRE-like protein 1	Kirrel	7.058992391	0.0005
Partitioning defective 3 homolog B	Pard3b	5.336486281	0.00002
Dendrin	Ddn	5.334060789	0.00003
Immunoglobulin-like domain-containing receptor 2	Ildr2	4.513838678	0.009
Src substrate cortactin	Cttn	4.005690317	0.0003
LIM zinc-binding domain-containing Nebulette	Nebl	3.646032083	0.003
Formin-binding protein 1-like	Fnbp1l	2.922884356	0.01
F-actin-capping protein subunit alpha-2	Capza2	2.817267174	0.01
V-type proton ATPase subunit E 1	Atp6v1e1	2.299927324	0.04
Calnexin	Canx	2.257874086	0.04
Myocardial zonula adherens protein	Myzap	2.087877712	0.00005

B



C

Annotated Cluster 1	Enrichment Score	Counts	P-Value
Cell Junctions	5.61	23	1.60E-09
Bicellular Junctions		12	1.00E-09
Protein Complex Scaffold		4	0.0038

Annotated Cluster 2	Enrichment Score	Counts	P-value
Cell-to-Cell Adherence Junctions	4.49	13	1.20E-06
Cell-Cell Adhesion		8	0.0086

Figure 5. Mass spectrometry analysis to uncover podocyte foot process proteome A) Protein table with Log_2 fold change and p values **B)** Plot of molecular abundance Log_2 FC vs -Log(p-value) **C)** Table of gene ontogeny identified from DAVID analysis representing counts and enrichment scores for top two annotated clusters

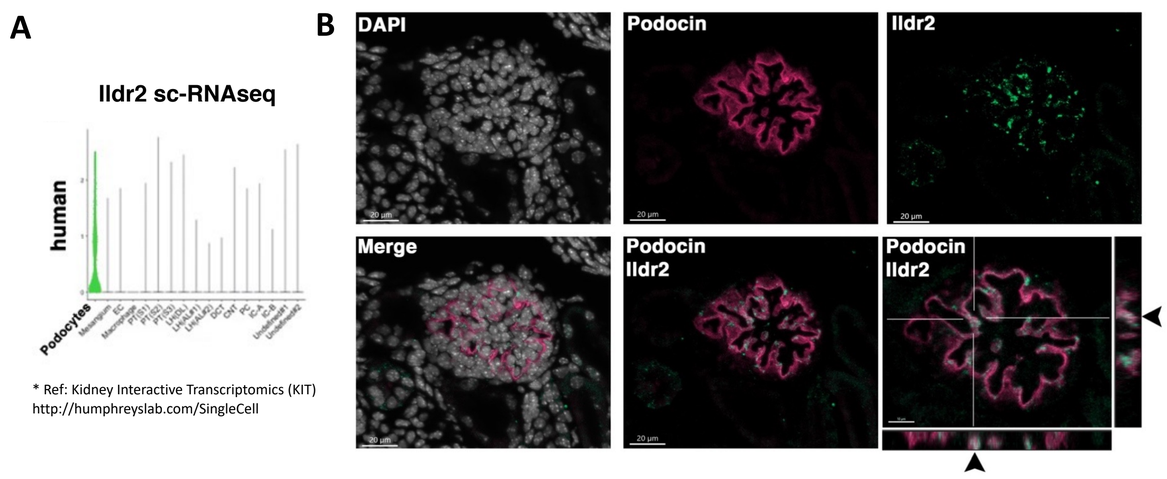


Figure 6. Expression of Ildr2 and podocin in mouse podocytes A) Single-cell RNAseq data identifies an Immunoglobulin-like domain-containing receptor 2 (Ildr2) is expressed in human podocytes. **B)** Immunofluorescence microscopy of Ildr2 and podocin in mouse podocytes. Frozen sections of mouse kidneys were stained with rabbit anti-Ildr2, rabbit anti-podocin, and DAPI to label nuclei. Black arrows indicate colocalization of podocin and Ildr2. The figure suggests that the two proteins colocalize in podocytes.

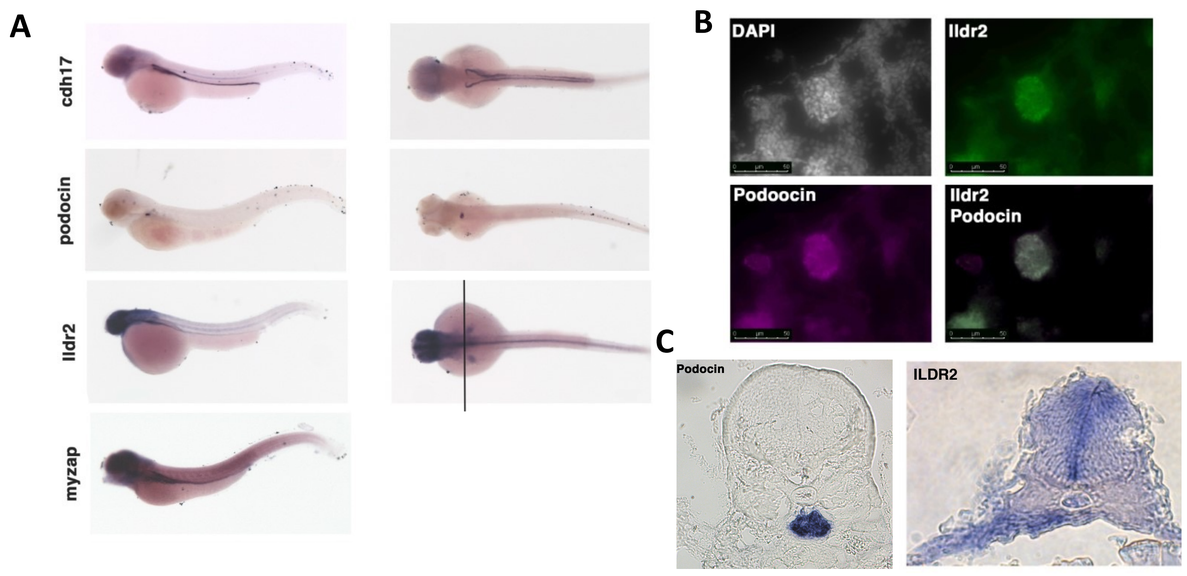


Figure 7. Expression of novel podocyte SD candidates in zebrafish A) Dorsal and lateral views of *in situ* hybridization of genes cdh17, podocin, Ildr2, and myzap in zebrafish pronephros at 48 hours post fertilization (hpf). **B)** Immunofluorescent analysis of adult zebrafish kidney, mesonephros, identifies Ildr2 signal within podocytes co-labeling with Podocin. **C)** Ildr2 *in situ* cross section denoted from horizontal line in (A) and Podocin.

Future Direction

We wish to take advantage of tissue-specific knockout of genes in order to further examine the purpose and function of proteins that localize in the podocyte and SD. We plan to utilize "Cre" and "Floxed" alleles to knockout genes specific to the kidney without affecting the gene(s) in other tissues. This will allow us to bypass potential problems where a full knockout of a POI could lead to lethality early in development.

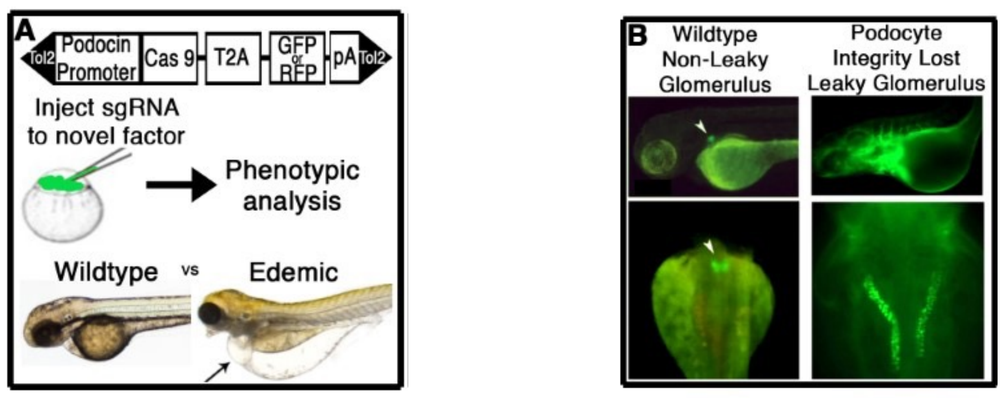


Figure 8. Our future approach for identifying and testing candidate factors that interact with Podocin . A) Tissue-specific knockout of a candidate factor will be achieved by utilizing the Podocin promoter to drive Cas9 expression. Loss of a candidate factor via tissue-specific knockout will be identified by phenotypic analysis of zebrafish for edema. Zebrafish that present edema will be analyzed via Transition Electron Microscopy (TEM) to detect for loss of podocyte foot process integrity. **B)** Immunofluorescence will be also utilized to determine whether podocyte integrity has been lost in zebrafish. If podocyte integrity is lost, fluorescent dye will leak into the tubules.

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