A Multi-layered Quantitative In Vivo Expression Atlas of the Podocyte Unravels Kidney Disease Candidate Genes

Graphical Abstract

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In Brief
The podocyte forms the most outer and essential part of the renal filter and restricts the passage of proteins from blood to urine. Rinschen et al. combine deep proteomic and transcriptomic data with protein dynamics from native mouse podocytes to reveal insights into podocyte biology and to identify candidate disease genes.

Highlights
- Deep proteome and transcriptome analyses of native podocytes unravel druggable targets
- Static and dynamic proteomics uncover features of podocyte identity and proteostasis
- Candidate genes for nephrotic syndrome were predicted based on multi-omic integration
- FAR1P1 is a previously unreported candidate gene for human proteinuric kidney disease
A Multi-layered Quantitative In Vivo Expression Atlas of the Podocyte Unravels Kidney Disease Candidate Genes

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SUMMARY
Damage to and loss of glomerular podocytes has been identified as the culprit lesion in progressive kidney diseases. Here, we combine mass spectrometry-based proteomics with mRNA sequencing, bioinformatics, and hypothesis-driven studies to provide a comprehensive and quantitative map of mammalian podocytes that identifies unanticipated signaling pathways. Comparison of the in vivo datasets with proteomics data from podocyte cell cultures showed a limited value of available cell culture models. Moreover, in vivo stable isotope labeling by amino acids uncovered surprisingly rapid synthesis of mitochondrial proteins under steady-state conditions that was perturbed under autophagy-deficient, disease-susceptible conditions. Integration of acquired omics dimensions suggested FARP1 as a candidate essential for podocyte function, which could be substantiated by genetic analysis in humans and knockdown experiments in zebrafish. This work exemplifies how the integration of multi-omics datasets can identify a framework of cell-type-specific features relevant for organ health and disease.

INTRODUCTION
Diseases involving glomeruli, the filtration units of the kidney, are a leading cause of chronic kidney disease (CKD), which affects approximately 15% of all Americans (Meguid El Nahas and Bello, 2018).
GFP, whereas all other viable glomerular non-podocyte cells expressing (FACS) (Boerries et al., 2013). Podocytes expressed hNPHS2Cre*mT/mG mouse using fluorescence-activated cell sorting (FACS) (Boerries et al., 2013). Podocytes expressed GFP, whereas all other viable glomerular non-podocyte cells expressing tomato red fluorescent protein (Figure 1A). A workflow for “deep mapping” of transcriptome, proteome, and proteome dynamics was applied to both podocytes and non-podocytes (Kulak et al., 2014). Quality control of the dataset demonstrated a clear separation between both samples (Figure S1). This approach identified, in total, more than 9,000 different proteins (Tables S1 and S2). We performed absolute quantification of 6,979 proteins using the intensity-based absolute quantification (IBAQ) approach (Table S2). This proteomics parameter is relative to copy numbers in a given sample (Köhli et al., 2014; Schwanhausser et al., 2011). Cytoskeletal proteins were highly represented in the dataset (Figure 1A). The dynamic range of the podocyte proteome copy numbers comprises seven orders of magnitude (Figure 1B). We mapped genes associated with focal segmental glomerular sclerosis or hereditary nephrotic syndrome on this dataset (Figure 1C; top 10 genes in Figure 1D; Bierzynska et al., 2015). These “disease-associated genes,” although only comprising 35 of 7,000 (0.5%) absolutely quantified proteins, contributed approximately 4% of the podocyte protein mass, with cytoskeletal proteins like Actn4 and Arhgdia contributing most to this amount (Figure 1D). Several other known podocyte genes (e.g., Trpc6) were identified but could not be quantified because of their low abundance. To estimate the absolute protein quantity based on the data and determine protein concentrations or copy numbers per cell, we applied a recently developed algorithm on our dataset (“proteomic ruler”) (Wisniewski et al., 2014). This provides a starting point to determine the stoichiometries of known protein complexes; for example, the prominent transmembrane slit diaphragm complex within native mouse podocytes, which comprises podocin-Neph1-Nephrin-Fat1 with a copy number ratio of \(10:2:1:0.5\) (Figure 1E), indicating that podocin is by far the most abundant slit diaphragm protein within podocytes (Graham et al., 2013).

Integration of Deep Transcriptomic and Proteomics Data

Protein abundance is determined by the rate of transcription but also a result of protein stability and posttranslational processing. To identify the determinants of podocyte protein copy number abundance, we performed comparison with a transcriptomic copy number analysis performed by mRNA sequencing. Our dataset was similar to previously published mRNA sequencing (mRNA-seq) analyses but demonstrated a greater depth upon analysis with the same parameters (Figure 2A; Table S3; Figure S2). Enrichment analyses demonstrated overrepresentation of several known pathways enriched on mRNA levels, among these extracellular matrix (ECM) synthesis and nephrin interactions (Figure 2B; Figures S2E–S2I; Brunskill et al., 2011). To unravel the correlation of transcriptomic data with proteomic data as a whole, iBAQ data were correlated with the transcriptomic (rpm) values, but the correlation was rather weak (Figure 2C). The correlation was stronger for podocytes compared with non-podocytes (Figure 2D). We checked which proteins were specifically stabilized or destabilized posttranscriptionally using 2D enrichment (Cox and Mann, 2012). This algorithm can be used to visualize statistically significant distributions of protein annotations in a 2D space between different “omics” datasets.
Cox and Mann, 2012). The analysis revealed that ECM protein expression was decreased as expected by the respective mRNA expression, which would be expected because of loss of podocyte-produced ECM proteins excreted as part of the glomerular basement membrane assembly (Figure 2E, blue). Proteins involved in controlling metabolism were largely stabilized (Figure 2E, magenta, green). These proteins included glycolysis gene products and proteins involved in amino acid biosynthesis.

Determination of Podocyte-Enriched Proteins
Most of the genes mutated in genetic forms of proteinuria or nephrotic syndrome encode for podocyte-enriched genes. Therefore, we next analyzed whether we could expand the list of known podocyte-enriched proteins compared with other glomerular cells. Using stringent statistical cutoff values for quantification, we found 551 podocyte-enriched proteins (Figure 3A; Table S2); among these were many gene products currently known to be associated with hereditary nephrotic syndrome (Sadowski et al., 2015). Figures S3 and S4A depict standardized stainings of 190 proteins that have a “medium” or “high” staining intensity in the human protein atlas (Uhlen et al., 2015). A systematic Medline search of these 190 proteins revealed that the majority were not explicitly linked to podocytes (Table S4). We performed statistical enrichment for overrepresentation of protein domains in all 551 proteins enriched in podocytes (Figure 3B). Significantly enriched protein domains were Fn3 and immunoglobulin G (IgG)-like domains (e.g., in Nephrin), PDZ domains (e.g., in ezrin and Par-3), i-set domains (e.g., in nephrin and VCAM/NCAM), CH domain (e.g., in actinin-4), and FERM domain proteins (e.g., in ezrin) or tetraspanins (e.g., CD151). On a global level, podocyte-specific proteins were significantly reduced for mitochondria as well as RNA and DNA binding proteins (Figure 3C) and enriched for signaling receptors, cytoskeleton-associated proteins, GTPase-associated proteins, membrane proteins, and (phospho)lipid-modifying proteins (Figure 3D).

To demonstrate that this resource may be used to find essential podocyte proteins, we analyzed the effect of knockdown of one candidate podocyte-enriched gene in Drosophila nephrocytes, an established model of podocyte function (Weavers et al., 2009; Zhang et al., 2013). We knocked down Tsp26A, the Drosophila homolog of TSP5 and TSP15, two newly identified podocyte-enriched proteins that are representatives of the protein class of tetraspanins (Figure 3D). This resulted in significantly decreased ANF-RFP uptake, indicating loss of filtration function (Figure 3E). We also found overt alterations in nephrocyte ultrastructure (Figure 3F), proving the importance of the tetraspanin protein class for nephrocyte function.

Podocyte protein homeostasis largely depends on signaling mediated via posttranslational modifications, such as phosphorylation, ubiquitylation, acetylation, and proteolytic processing (New et al., 2014). Pinpointing podocyte-specific signaling molecules could help us to understand podocyte physiology and target podocyte signaling pharmacologically or genetically. Table 1 summarizes unanticipated observations of signaling molecules enriched in mouse podocytes; among these were...
several druggable molecules. (For a full list of podocyte enriched
kinases, phosphatases, ubiquitin ligases, proteases, signaling
receptors, actin binding proteins and transcription factors, see
Table S5). We also mapped the 551 podocyte-enriched proteins
against known protein interaction databases to obtain a network
and central nodes among these podocyte-enriched proteins
(Cerami et al., 2010). The proteins RhoA, Actin, and Grb
and the nephrin-regulating kinase Src had most edges within these
networks (Figure S4B); all four have reported relevance for podo-
cyte biology (Harita et al., 2008; New et al., 2014; Schmieder
et al., 2004), suggesting a central role for these proteins.

Comparison of the Deep Native Podocyte Proteome with
the Cultured Podocyte Proteome

We asked whether this unique obtained podocyte proteome
resource would help us to investigate the strengths and weak-
nesses of in vitro immortalized podocyte cell cultures, a commonly used tool in
podocyte research. To this end, we
compared the dataset with a similar, pre-
viously published deep quantitative pro-
tomic mapping of a mouse podocyte
cell line (Rinschen et al., 2016b). We could
identify 6,193 proteins in both samples,
representing a significant overlap be-
tween both samples (Figure S4C). Clus-
tering analysis of normalized expression
values revealed significant differences
between native podocytes and the
in vitro
cell line irrespective of the differen-
tiation status (33°C/C14°C = undifferentiated or
37°C/C14°C = differentiated; Figure S4D). Clus-
tering analysis revealed eight major clusters. Three clusters contained proteins
with high abundance in cell lines compared with the native podo-
cytes and were enriched for proteins related to the actin cyto-
skeleton, including stress fibers and focal adhesion proteins
( Figure 4A). Three clusters contained proteins related to phospholipid metabolism
and tight junctions. Two clusters showed differences between
33°C and 37°C and native cells; these were, as expected
(Rinschen et al., 2016b), related to the cell cycle (Figure 4C).
Moreover, disease-causing proteins such as nephrin and podo-
cinin or podocyte markers such as WT1 were not at all or only very
weakly expressed in podocytes in culture compared with the
in vivo dataset.

This comparison of podocyte proteomes from cell culture and
from native tissue suggested that podocyte cell cultures have an
increased abundance of stress fiber-associated proteins and
focal adhesions. We postulated that some of these differences may have been a cell culture artefact because of the mechanical properties of the plastic dish on which these cells were cultivated. As a proof of principle, we cultured podocytes on soft matrix with an elastic modulus of 12 kPa and compared their proteome with podocytes grown in plastic dishes (in the range of gigapascals). This elastic modulus (“stiffness”) of 12 kPa is similar to that of a skeletal muscle cell, a mechanically challenged cell (Gilbert et al., 2010; Janmey and Miller, 2011). Using quantitative proteomics, we found vast differences in the podocyte proteome depending on the matrix on which they were grown (Figures S5A and S5B). On a global level, actin filament proteins and stress fiber proteins were significantly depleted by culturing podocytes on the “soft” matrix, which made them, in this regard, “more similar” to the native podocyte (based on the log2 ratio of podocyte/non-podocyte protein expression) (Figure 4D). A similar picture arose when human podocytes were cultured on soft matrices. The changes in the proteome were larger (Figure S5), and the analysis confirmed again the loss of stress fiber protein expression on the soft matrices (Figure 4E; p and false discovery rate (FDR) < 0.05 in a Fisher’s exact test).

Analysis of Podocyte Proteome Dynamics and Integration with Deep Proteomics Mapping

To conclude, our data quantitatively describe expression levels in the podocyte proteome. However, these data did not yet cover an important, rather unexploited component of global proteostasis regulation in vivo: the dynamic synthesis and turnover of proteins. To approach this, we used a mouse model of in vivo stable isotope labeling (Table S6). To this end, adult mice (10 weeks of age) were fed for 1, 2, and 3 weeks with a diet exclusively containing heavy isotope-labeled lysine. The incorporation of this essential amino acid should correlate with the dynamic synthesis rate (although these data do not strictly correlate with a protein “half-life” because of intracellular amino acid recycling (Kruger et al., 2010).
et al., 2008). Still, proteins synthesized rapidly should exhibit a high heavy amino acid incorporation rate (and a high heavy/light [H/L] ratio), whereas stable, “long-lived” proteins with slow translation should have a lower incorporation rate (Savas et al., 2012). To see which proteins incorporate isotopes at a similar speed, we performed hierarchical clustering of H/L ratios over the labeling time course. This analysis revealed four major clusters of proteins (Figures 5A and 5B). Gene ontology (GO) term analysis of these four clusters revealed that endoplasmic reticulum (ER)-associated proteins (cluster 4) were particular “dynamic” compared with the nuclear proteins, in particular histones (cluster 1), which only showed minimal increases in the H/L ratio (Figure 5C), consistent with previous studies (Toyama et al., 2013). There was almost no correlation between iBAQ values and H/L ratios (correlation coefficient $R^2 = -0.01$) (Fig. S6A).

When comparing podocyte H/L ratios with those of co-sorted non-podocyte cells, we observed that podocyte proteins incorporated more slowly than the parallel sorted non-podocyte cells (Figure 5D). 2D GO analysis revealed that cytoskeletal and mitochondrial proteins had a particularly high protein turnover in podocytes compared with non-podocyte cells (Figure 5E). We also analyzed an intact whole kidney protein sample from an
Figure 4. Comparison of the Deep Native (Isolated from Living Mice) and Cultured Podocyte Proteome Enables Functional Analysis of Cell Culture-Induced Proteome Artefacts

(A) Protein clusters were defined based on normalized intensities as depicted in Figure S4 C–D. Shown are clusters that have a higher intensity in cultured podocytes compared with the native podocyte proteome. Distance to the mean is color-coded in each cluster. GO terms significantly overrepresented in each cluster are depicted (Fisher’s exact test, p values corrected with FDR < 0.05).

(B) Three clusters have a lower intensity (and abundance) in the podocyte proteome compared with the cultured podocyte proteome. GO terms significantly overrepresented in each cluster are depicted.

(C) Two clusters have different intensities in undifferentiated and differentiated podocytes.

(D) Mouse podocytes were seeded on matrices with 12 kPa, and the proteome was compared with control cell culture dishes. See Figure S5 for details regarding the dataset. The 2D GO enrichment score of fold change on soft matrix (log2 12 kPa/control) cell culture is plotted against the score for podocyte-enrichment (log2(podocyte/non-podocyte)) (Figure 3). The data demonstrate a significant decrease of stress fiber proteins under both conditions (soft versus stiff matrix and native podocyte versus non-podocyte). Significantly changed terms are plotted (FDR < 0.05).

(E) Human podocytes were seeded on matrices with 12 kPa, and the proteome was compared with control cell culture dishes. See Figure S5 for details regarding the dataset.
independent parallel study (Figures S6B and S6C), confirming that mitochondrial proteins were incorporated relatively faster in podocytes compared with the whole kidney. However, this comparison did not respect overall protein amounts, and protein amounts are significantly different between podocytes and non-podocytes (Figure 3A). Thus, a mere relative incorporation rate is not sufficient to estimate how much "energy" a podocyte spends to maintain the specific protein compared with another cell type; this would crucially depend on the copy numbers and, as an ancillary factor, on the size of the protein. Therefore, we corrected the incorporation rates by total protein abundance and size (Experimental Procedures). The analysis revealed that actin-binding proteins and cytoskeletal proteins incorporated heavy amino acids markedly stronger (Figure 5F). Interestingly, four proteins associated with hereditary podocyte disease were in the upper quartile of most dynamic proteins, among these podocin (Nphs2), Cd2ap (Cd2ap), and actinin-4 (Actn4) (Figures 5F and 5G) and Magi2 (Bierzynska et al., 2016; Figure 5F). The number of amino acids incorporated into mitochondrial proteins was not different in podocytes compared with non-podocytes (Figure 5G), which could be explained by low mitochondrial mass (Figure 3) but high turnover of these organelles (Figure 5B). These data indicate that some of the podocyte’s unique proteotypic features are partially compensated by increased protein synthesis and, potentially, proteostasis.

An unexpected finding was that podocytes have high mitochondrial protein synthesis but an overall low protein copy number of mitochondrial proteins. We asked whether this balance would tilt further when genetic manipulation of podocyte proteostasis was performed. We performed quantitative pulse labeling of amino acids in young control mice as well as in young mice with podocyte-specific knockout of autophagy-related 5 (ATG5 podocyte specific knockout [pKO]), a major positive regulator of autophagy in general and especially in podocytes (Hartleben...
Figure 6. Identification of Candidates for Human Nephrotic Syndrome Disease-Causing Genes

(A) Clustering analysis (t-SNE) of 6,700 proteins for which absolute protein expression, relative protein expression, mRNA expression, and tissue-specific mRNA expression were available.

(B) Heatmap analysis of expression of disease genes and one previously unreported candidate gene, FARP1, as well as five candidates from a previous study (Yu et al., 2016).

Figure continued on next page.
et al., 2010; Table S6). The ATG5 pKO allele makes podocytes extremely susceptible to damage (Hartleben et al., 2010). We observed that ATG5 knockout podocytes had a significantly lower incorporation of endoplasmic reticulum proteins, a finding expected from a defect in autophagy, and, surprisingly a significantly higher incorporation of mitochondrial proteins compared with control podocytes (Figure 5H). This was consistent with 2D GO enrichment time points after different time points (Figures S6D and S6E). Isotope-labeled lysine incorporation in non-podocyte cells was not changed dramatically across both conditions (Figure 5I). In conclusion, these data delineate a specific perturbation in isotope incorporation in podocytes, specifically affecting mitochondria.

**Multi-omics Integration Reveals Candidate Genes for Podocyte Function in Humans**

Finally, we asked whether the integration of genomic and proteomic analyses could reveal candidate genes essential for podocyte function. To this end, we performed clustering analysis of z-normalized relative mRNA expression levels, relative protein expression levels, absolute protein expression levels, and tissue specificity of podocyte mRNA levels. We found that disease-associated genes in slit diaphragm and actin-related processes were largely defined by very high Z scores in all of the four parameters and clustered close to each other (Figures 6A and 6B). Subsequently, we defined 280 genes based on each of these criteria (Table S7) and screened exome sequencing of 430 families with hereditary nephrotic syndrome for homozygous mutations in these genes. As a result, we identified a homozygous mutation in the gene FARP1 in one family with nephrotic syndrome (Table S7). This candidate gene, and the genes identified in a recent population-based study (Yu et al., 2016), clustered close to known disease genes (Figure 6A), and all shared high Z scores in at least three of the four parameters analyzed (Figure 6B). From a genetic perspective, several factors suggest potential pathogenicity of the identified allele: the mutation affects amino acid residues that are highly conserved among orthologs across evolution; in a control database of 80,000 predominantly healthy individuals (Exome Aggregation Consortium [ExAC]), the minor allele frequency of either allele is below 0.01%, and it has not been reported in the homozygous state; and the allele segregates with the affect status within the family (Figure 6C). The mutated residues were highly conserved and the mutation localized in a functional PH domain of the protein, and histology and electron microscopy showed clear signs of podocyte dysfunction within the affected individual (Figures S7A–S7E). A second patient with previously non-identified FARP1 variants was identified in a second cohort of 700 patients with nephrotic syndrome (patient was heterozygous for T789M, and c.-20T > A); however, it was unclear whether the alleles segregated with the phenotype because the DNA of family members could not be sequenced. The FARP1 gene newly identified in this study also localized predominantly to glomeruli and their precursors in development and kidneys of newborn mice (Figures 6D and 6E). In addition, knockdown of the farp1 mRNA in zebrafish larvae resulted in a phenotype of pericardial edema as well as proteinuria, indicating loss of glomerular barrier function in D. rerio (Figure 6; Figures S7F and S7G).

**DISCUSSION**

Postmitotic specialized cell types, such as neurons or podocytes, are particularly vulnerable to age-dependent degeneration. In fact, the podocyte has emerged as a central gatekeeper to prevent proteinuria and renal failure, two unmet and frequent clinical symptoms associated with dramatically increased cardiovascular risk. Interrogation of individual cell types at multiple omics levels can yield insights into both essential molecular components and preventive strategies. Here we gained a near-comprehensive view of the static and dynamic podocyte proteome and transcriptome by applying recently developed technologies (Kulak et al., 2014). Single-cell podocyte sorting previously enabled only incomplete in vivo analysis of the podocyte proteome (Boerries et al., 2013; Rinschen et al., 2015a, 2016a). At least 10,000 proteins are suggested to be expressed in a cultured cell line (Kulak et al., 2014; Mann et al., 2013; Nagaraj et al., 2011), and a similar number of proteins has been shown to be expressed in native neurons (Sharma et al., 2015). Here we demonstrate that such a comprehensive atlas of protein and mRNA expression datasets now allows distilling molecular characteristic features of podocytes, ultimately up to the prediction of candidate genes associated with human disease. Figure 6G gives an overview of the findings in this study.

Podocytes express a high abundance of actin-binding proteins and proteins known to be localizing to the leading edge of the cell (Figures 1, 2, and 3; Table S5). Expressed in simplified terms, many of these proteins oppose the formation of stress fibers by promoting actin branching to promote a “motile” phenotype (Higgs and Pollard, 2001). The leading edge is a long-established model to study the cell biology of podocyte foot processes, and eventual slit diaphragm signaling (Garg and Holzman, 2012; Garg et al., 2010; Moeller et al., 2004), and based on these global proteomic data, this paradigm could be further expanded. This study discovered signaling molecules enriched in podocytes, which may be directly translated to novel experimental studies. Several of the proteins, but by far not all of them, have already been studied in podocytes and have been linked in part by dramatic phenotypes in conditional and targeted mouse models (e.g., protein kinase C) (Huber et al., 2009) or human patients (e.g., MAGI2; Bierzynska et al., 2016). A
significant fraction of these proteins, however, has not been extensively studied and will require further consideration (Table 1; Table S4); for instance, specific members of the tetraspanin family, which were confirmed in Drosophila nephrocyte studies as highly conserved functional regulators of filtering cells (Sachs et al., 2012; Figure 3).

In addition to using these data as a resource, one may also delineate “systems-level” features of molecular podocyte identity. To demonstrate this approach in principle, we delineated differences to the proteome of a commonly used immortalized mouse podocyte cell culture line (Rinschen et al., 2016b). Cultured podocytes are widely used to investigate podocyte damage and serve as a model to study the regulation of the cytoskeleton and many other aspects of podocyte biology (Lee et al., 2015a; Shankland, 2006; Yu et al., 2013). Surprisingly, the systems-level comparison of proteome-wide expression data (Figure 4) revealed that proteins involved in stress fiber formation are among the strongest dysregulated processes in cell cultures, a finding that could be caused by stiffness of the cell culture dish (Gilbert et al., 2010). As a proof-of-principle experiment, the cultivation of either human or mouse podocyte cells on soft matrices resulted in a major shift in the proteome, which partly reduced this actin-related cell culture artefact (Figure 4). This is consistent with observations that podocytes strongly react to other mechanical cues, such as hydrostatic pressure (Kriz and Lemley, 2015), stretch (Durvasula et al., 2004; Kim et al., 2013; Petermann et al., 2005), or membrane curvature (Inoue and Ishibe, 2015), in vitro and in vivo (Hu et al., 2017; Rinschen et al., 2017). Cultivation on soft matrices does not, however, increase the expression of podocyte-specific actin binders or podocyte membrane proteins, which are only minimally expressed under both conditions.

Mitochondrial proteins are rarely expressed in podocytes (Figure 3), but the synthesis of these proteins (as measured by in vivo stable isotope pulse labeling) is faster than in non-podocytes. Therefore, the podocyte has an overall low abundance of mitochondrial proteins but high mitochondrial resynthesis and, assuming a steady-state equilibrium, also turnover. This may be a surrogate readout of predominant mitochondrial degradation (e.g., by mitophagy) in podocytes compared with other glomerular cell types. Consistently, podocytes show protein-level enrichment for the ubiquitin ligase Parkin2 (log2 fold change 2.33), a key molecule controlling mitophagy (Narendra et al., 2008; Table S2). Interestingly, when knocking out ATG5, a protein essential for autophagy (Hartleben et al., 2010), the podocyte reacts, when still intact and functional, with even further increased synthesis of mitochondrial proteins (Figure 5H). One could speculate that this finding may be caused by increased alternative autophagy and mitophagy, a process significantly increased in the absence of ATG5 (Hirota et al., 2015). In fact, mediators of alternative autophagy (e.g., Rab9 and Becn1) are abundantly expressed in podocytes but not significantly enriched (Lee et al., 2015b; Table S2). Although these findings need further corroboration, these data demonstrate the feasibility to dissolve dynamic proteostatic shifts in vivo by integrating dynamic and static protein quantifications and cutting through the various dimensions of podocyte proteostasis.

In summary, these data provide a unique resource and starting point for the next level of systematic understanding of podocyte identity, function, and glomerular disease mechanisms, including a systems view of podocyte proteome dynamics (Figure 6G). Using a multidimensional analysis, we showed that a majority of podocyte disease genes (mainly slit diaphragm- and actin-related genes) have rather high relative and absolute protein expression as well as podocyte-specific mRNA expression (Figures 6A and 6B). This opens the door for a potential integrative “reverse genetic” approach to predict functionally relevant candidate genes associated with human podocyte diseases (Figure 6; Table S7) and prioritize them for further functional testing, in particular when more advanced bioinformatics methods are utilized and further data (e.g., transcriptome data from patient cohorts) are integrated. The concept presented here will be advantageous for understanding cell-type-specific function in health and disease.

**EXPERIMENTAL PROCEDURES**

**Animals**

Gt(Rosa)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J mice were purchased from The Jackson Laboratory (Bar Harbour, Massachusetts, USA) (Muzumdar et al., 2007), and Tg(NPHS2-cre)295Lbh mice were a generous gift from M. Möller (University of Aachen, Aachen, Germany). Use of Atg5flox mice has been reported previously (Hartleben et al., 2010). In general, Gt(Rosa)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, Tg(NPHS2-cre)295Lbh mice (for deep proteome, RNA-seq, and pulsed in vivo stable isotope labeling experiments) as well as Gt(Rosa)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, Tg(NPHS2-cre)295Lbh;Atg5flox mice (pulsed in vivo stable isotope labeling experiments) were on a mixed genetic background (Sv129/C57Bl6/Icr). Breeding and genotyping was done according to standard procedures. Mice were housed in an SPF facility with free access to chow and water and a 12-hr day/night cycle. All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals and were approved by local authorities (Regierungspräsidium Freiburg X12/06J, G11-38, and G11-34). Glomeruli and, subsequently, podocytes were isolated using essential the same method as described previously (Boerries et al., 2013). For details, see Glomerular Isolation and Podocyte Preparation in the Supplemental Experimental Procedures.

**Liquid Chromatography-Tandem Mass Spectrometry Acquisition**

For details regarding sample preparation of native podocytes for proteomic analysis, see the Supplemental Experimental Procedures. Analysis of peptides was performed using a quadrupole-orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific, Bremen) coupled to a nano-liquid chromatography (nLC) device. The mass spectrometers were calibrated weekly. Briefly, peptides were separated by nLC using a 4-hr gradient (flow rate, 200 nL/min). Peptides were separated on an in-house packed 50-cm column with 1.7-µm C18 beads (Dr. Maisch). Ascending concentrations of buffer B (80% acetonitrile, 0.1% formic acid) over buffer A (0.1% formic acid) were used. Then peptides were sprayed into the mass spectrometer by electron spray ionization. The acquisition parameters for the mass spectrometer were as described previously (Bartram et al., 2016). 1E6 was the AGC target for MS1. The resolution was 70,000 (mass range, 200–1200 m/z) for MS1, and Tandem mass spectrometry (MS/MS) spectra of the top 10 most intense peaks were obtained by higher-energy collisional dissociation fragmentation. Resolution for the MS/MS spectra was 35,000 at 200 m/z, and AGC target to 5E5.

**Cell Culture and Proteomics Analysis**

Validated cell lines were regularly tested for mycoplasma using a commercial kit (VENOR, Sigma). Human podocytes (Saleem et al., 2002) were cultured in dishes as described previously at 33°C (Rinschen et al., 2016a). They were seeded on collagen-coated soft matrices (Matrigen) for 2 days (passage
~20). Similarly, mouse podocytes (at 33°C) (Griffin et al., 2004) were cultured in coated Primaria cell culture dishes (Falcon) as described previously and seeded on collagen-coated soft matrices (Matrigen) for 2 days. For details regarding sample preparation, proteomics, and bioinformatics analysis, see the Supplemental Experimental Procedures.

**Pulsed In Vivo Stable Isotope Labeling**

Stable isotope labeling of animals was performed as described previously by Krüger et al. (2008). The Lys(0)-stable isotope labeling by amino acid in cell culture (SILAC)-Mouse control (12C6-lysine, “light”) and Lys(6)-SILAC-Mouse SILAC (13C6-lysine, 97%, “heavy”) mouse diets were purchased from Silantes (Martinsried, Germany). For details regarding sample preparation and proteomics of in vivo stable isotope-labeled tissue, see the Supplemental Experimental Procedures.

**Statistics**

For quantitative data, statistical tests (two tailed unpaired t test, Kolmogorov-Smirnov test, and two-way ANOVA test) were performed where appropriate and where indicated. In general, p < 0.05 was considered significant. For large-scale data, correction for multiple testing was performed as described in the respective bioinformatics method sections.

**Study Participants, Whole-Exome Sequencing, Multi-gene Panel Testing, and Mutation Calling**

We obtained blood samples and pedigrees following informed consent from individuals with steroid resistant nephrotic syndrome (SRNS) or their legal guardians. Approval for human subject research was obtained from the institutional review boards of the University of Michigan and Boston Children’s Hospital. For details regarding whole-exome sequencing, multi-gene panel testing, and mutation calling, see the Supplemental Experimental Procedures.

**Model Organism Experiments**

The detailed methods for functional analysis of zebrafish and Drosophila proteinic phenotypes as well as mouse in situ hybridization experiments can be found in the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.04.059.

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**AUTHOR CONTRIBUTIONS**

M.M.R., M.G., F.G., T.B., and T.B.H. designed the research studies. M.M.R., M.G., F.G., S.Z., M.H., O.K., M.Z., D.A.B., S.D., C.P., C.T., H.Y.G., and V.K. conducted experiments. G.D., M.P., D.A.B., E.K., C.B., and F.H. acquired genetic data. M.S. and P.S. performed zebrafish experiments. M.M.R., M.G., B.S., M.B., H.B., J.D., and T.B.H. analyzed data. M.K. and J.D. provided reagents and novel tools. M.M.R., M.G., F.G., T.B., and T.B.H. wrote the manuscript.

**DECLARATION OF INTERESTS**

F.H. is a founder of Goldfinch-Bio and a member of its scientific advisory board. C.B. and E.K. are employees of Bioscientia/Sonic Healthcare. C.B. holds a part-time faculty appointment at the University of Freiburg.

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