Altering a Histone H3K4 Methylation Pathway in Glomerular Podocytes Promotes a Chronic Disease Phenotype

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Abstract

Methylation of specific lysine residues in core histone proteins is essential for embryonic development and can impart active and inactive epigenetic marks on chromatin domains. The ubiquitous nuclear protein PTIP is encoded by the Paxip1 gene and is an essential component of a histone H3 lysine 4 (H3K4) methyltransferase complex conserved in metazoans. In order to determine if PTIP and its associated complexes are necessary for maintaining stable gene expression patterns in a terminally differentiated, non-dividing cell, we conditionally deleted PTIP in glomerular podocytes in mice. Renal development and function were not impaired in young mice. However, older animals progressively exhibited proteinuria and podocyte ultra structural defects similar to chronic glomerular disease. Loss of PTIP resulted in subtle changes in gene expression patterns prior to the onset of a renal disease phenotype. Chromatin immunoprecipitation showed a loss of PTIP binding and lower H3K4 methylation at the Ntrk3 (neurotrophic tyrosine kinase receptor, type 3) locus, whose expression was significantly reduced and whose function may be essential for podocyte foot process patterning. These data demonstrate that alterations or mutations in an epigenetic regulatory pathway can alter the phenotypes of differentiated cells and lead to a chronic disease state.

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Introduction

The process of embryonic development determines the differentiated state of all cells by establishing unique gene expression patterns, or signatures, for individual cell types that define their phenotypes. Once a differentiated state is established, it is difficult to erase that epigenetic imprint and reprogram the cell towards a different cell lineage or phenotype. Although reprogramming can be forced by nuclear transplantation [1] or by the expression of Oct4 and accessory factors [2,3], the low efficiency of these processes speaks to the inherent stability of a differentiated cell. Gene expression patterns must be established and maintained by compartmentalizing the genome into active and inactive regions, which is thought to occur through the covalent modifications of DNA and its associated nucleosomes. Such modifications include DNA methylation of CpG islands and methylation, acetylation, and ubiquitination of histone tails, all of which are thought to determine chromatin structure and accessibility [4,5]. This epigenetic code is thus imprinted upon the primary genetic code during embryonic development to help establish cell lineages and restrict fate.

The genetics and biochemistry of histone modifications have been well studied in a variety of model organisms and developmental contexts. Genes of the Polycomb and Trithorax families encode proteins that are required for methylation of different histone lysine residues and often correlate with gene silencing or activation, respectively [6–9]. Many Trithorax group proteins, such as Drosophila TRX and human KMT2A (MLL), are histone H3 lysine 4 (H3K4) methyltransferases (KMTs) and are essential for maintaining gene expression patterns in diverse organisms. Recently, we discovered a novel co-factor, PTIP (Pax Transactivation-domain Interacting Protein), which is encoded by the Paxip1 gene. The PTIP protein co-purifies with the mammalian lysine methyltransferases KMT2B and KMT2C (formerly ALR and MLL3), is broadly expressed, and is essential for embryonic development [10–12]. At least in one case, PTIP is able to recruit the KMT2B complex to a developmental DNA binding protein in a locus specific manner [13]. Loss of PTIP function in the mouse results in gross developmental effects at gastrulation, with reduced levels of global H3K4 di- (me2) and trimethylation (me3) observed [13,14]. In cultured mouse embryonic stem cells, PTIP is needed to maintain pluripotency, Oct4 expression, and normal levels of H3K4 trimethylation [15]. Similarly, in neuronal stem cells, differentiation is abrogated and levels of H3K4 methylation are reduced in tissue specific PTIP knockouts [13]. In mouse embryo fibroblasts, loss of PTIP blocks...
differentiation by inhibiting PPARγ and C/EBPβ activation and H3K4 methylation at their respective promoters [16]. Similarly, the Drosophila homologue of PTIP is also essential for development, epigenetic control of gene expression, and global histone H3K4 methylation [17].

During cell division, patterns of histone methylation must be inherited by daughter cells such that the cellular phenotype is maintained. For repressive histone methylation marks, such as histone H3 lysine 27, the EED (Embryonic Ectodermal Development) protein is thought to bind and recruit the Polycomb Repressor Complex 2 to regulate and maintain gene silencing after mitotic cell division [18,19]. For highly expressed genes, the KMT2A (MLL1) protein associates with promoter regions on condensed mitotic chromatin and is required to reactivate such genes after cell division [20]. These data suggest a model whereby histone methylation patterns are replicated during mitosis, but do not address the necessity for maintaining epigenetic modifications in terminally differentiated, non-dividing cells. Furthermore, changes in the expression of epigenetic regulatory genes have been reported in a variety of cancers [21] and disease states [22], but whether these are the cause or the result of disease remains to be determined.

To address the necessity of H3K4me3 in a stable non-dividing cell type, we utilized a Podocin-Cre transgenic driver to delete PTIP in the glomerular podocyte, a highly specialized and architecturally distinct cell type that establishes the kidney filtration barrier. Podocytes are clinically relevant cells whose properties and expression profiles change in glomerular diseases and in older animals [23]. While the ubiquitous expression of PTIP, its role in H3K4 methylation, and its necessity in development and differentiation are all well established, whether PTIP deletion in terminally differentiated cells can induce changes in the pattern of H3K4me3 and gene expression has not been demonstrated. We show that loss of PTIP results in changes in the transcriptional profile of terminally differentiated podocyte cells, which ultimately leads to a chronic glomerular disease phenotype. Among the most affected is the neurotrophin receptor encoding gene Ntrk3, whose function had not been previously studied in podocytes. Our results demonstrate a maintenance function for PTIP-mediated H3K4 methylation and identify a novel role for Ntrk3 in podocyte foot process patterning.

Results

Generation of a Podocyte-Specific Paxip1 Deletion

To specifically knock out PTIP protein in fully differentiated mouse podocytes, we utilized both floxed (fl) and conventional null (−) alleles of Paxip1 and a Cre driver strain specific for glomerular podocytes. The Paxip1fl/−:CreNphs2 mice were crossed to Paxip1fl/fl animals to generate Paxip1fl/fl or Paxip1fl/+ with or without CreNphs2. The CreNphs2 mice utilize the Nphs2 promoter to express Cre recombine only in late developing and mature podocytes [24,25]. The resulting progenies were born in the expected Mendelian ratios and did not show any gross kidney defects during the first 4 weeks of life (data not shown). For simplicity, we will refer to the mice as either PTIP−/− or Paxip1fl/−:CreNphs2; Paxip1fl/fl; CreNphs2, or Paxip1fl/−; Paxip1fl/+, or Paxip1fl/+. PCR analysis indicated that recombination occurred at the Paxip1 locus in DNAs isolated from kidneys but not in DNAs from tails (Figure 1A). Previous work established that the Paxip1− allele produces normal levels of protein, but Cre-mediated excision of exon 1 and the promoter region results in complete absence of PTIP protein, essentially creating a null allele [13,15]. The specificity of the Cre driver strain was confirmed by crossing CreNphs2 mice to the Rosa26-LacZ reporter mice (Figure 1B). In 1 month old kidneys, lacZ expression was restricted to the glomerulus only, indicating efficient Cre mediated excision at this time. Immunostaining for PTIP and the podocyte marker WT1 also confirmed that PTIP protein levels were reduced only in the podocyte cells and not the mesangial or endothelial components of the glomerular tuft (Figure 1C).

Previous work showed that a loss of PTIP function results in reduced levels of total H3K4me3 levels in embryos and cultured cells [13–17]. To test whether podocytes showed reduced H3K4me3, we stained kidney sections with antibodies specific for this modification (Figure 1D). Many podocytes were observed with reduced signal intensity by integrating a fixed area over the nuclei of both podocytes and other cell types (WT1+). The resulting progenies were born in the expected Mendelian ratios and did not show any gross kidney defects (Figure 1E). Podocytes were co-stained with WT1 antibodies. The ratio of podocyte signal (WT1+) to other cell types (WT1−) was calculated by counting at least 6 cells of each type per glomerulus. The ratios from at least 8 glomeruli were averaged for each genotype and shown to decrease by more than 20% in PTIP−/− kidneys compared to PTIP+/+ controls (p<0.01). These data confirmed that the specific deletion of PTIP in the podocytes correlates with a reduction in H3K4me3 in this cell type.

Development of a Chronic Glomerular Disease Phenotype

Podocytes play a critical role in the establishment and maintenance of the glomerular filtration barrier. Interdigitated podocyte foot processes cover the glomerular basement membrane and form specialized junctions, called slit diaphragms, which create a highly selective barrier that filters small and negatively charged proteins and solutes from the blood to the urinary space. Damage to or loss of podocytes impairs the filtration barrier and results in increased rates of excretion of high molecular weight proteins, such as albumin, into the urine. Thus, we checked mice for proteinuria beginning at 1 month of age (Figure 2A). At 1 month, low levels of albumin were detected in the urine but these were not significantly different between PTIP−/− and
Figure 1. Generation of a Podocyte-Specific Paxip1 Deletion. A) PCR genotyping with primer pairs specific for the excised, null allele indicates Paxip1 excision only in the kidney DNA and only in mice carrying the CreNPHS2 transgene. B) Enzymatic staining for β-galactosidase activity (blue) in kidney sections from 1 month old mice with the indicated genotypes. C) Immunostaining for WT1 (green) and PTIP (red) in glomeruli at 3 months of age show reduced PTIP signals in the WT1 positive cells (arrows) of PTIP− kidneys compared to PTIP+ control littermates. The overlays were counterstained with DAPI to mark all nuclei. Thus double positives (WT1 and PTIP) are light purple whereas single positives (WT1 only) are green. D) Immunostaining for H3K4me3 and WT1 in kidneys of 3 months old PTIP+ and PTIP− mice. Note reduced intensity of podocyte cells (arrows) in PTIP− kidneys. E) Bar graph showing the signal ratio (WT1+/WT1−) of H3K4me3 expression in PTIP+ and PTIP− kidneys, with p < 0.01.
PTIP− animals. However, by 3 months of age the PTIP− mice showed significantly higher levels of albumin in the urine and these levels increased further at 6 and 12 months. The urine albumin to creatinine ratio (ACR) provides a quantitative assay that correlates with filtration barrier integrity. No significant differences were observed at 1 month (Figure 2B). However, by 3 and 12 months, ACR were 10 and 30 fold respectively in urines of PTIP− animals compared to PTIP+ mice. Mice that carried the CrePaxip1fl/+ genetic background did not show any renal abnormalities at 12 months (data not shown), consistent with many published reports that have used this particular Cre driver strain [25–28].

Renal pathology was characterized by light microscopy at 1, 3, and 12 months of age. Standard Masson’s Trichrome and Periodic-Acid-Shiff stainings revealed significant sclerosis and matrix deposition in 12 month old glomeruli from PTIP− animals (Figure 2C). However, 3 month old kidneys did not show significant differences for most glomerular sections, at the light microscopy level, although evidence of limited matrix expansion could be observed in a small number of glomeruli of PTIP− kidneys. In 12 month old kidneys, significant interstitial fibrosis and protein filled cysts were also observed (Figure 2D). These are likely to be secondary effects due to the glomerular pathology.

Glomerular pathology and increased albuminuria can be the direct result of podocyte death [29]. Thus, we used a variety of markers to characterize the glomerular architecture and the numbers of podocyte cells at various ages to insure that the phenotype of the PTIP− mice was not just the result of early podocyte cell death. Immunostaining with WT1, Nephrin, and Podocin antibodies enabled us to determine the podocyte numbers, as average per mid-cross section, and to indirectly assess the integrity of the slit diaphragm (Figure 3). The number of WT1 positive podocytes was not significantly different between PTIP+ and PTIP− glomeruli at 1 or 3 months of age. At 6 months, PTIP− glomeruli had slightly fewer podocytes and by 12 months, the number of podocytes was half that of the PTIP+ littermates. Immunostainings for podocyte markers such as WT1, Nephrin, and Podocin did not reveal dramatic differences at 1 or 3 months, despite the increase in proteinuria, although some discontinuous staining could be seen with Podocin antibodies in PTIP− glomeruli (Figure 3B). Consistent with this data, TUNEL staining for apoptosis did not reveal differences between PTIP+ and PTIP− kidneys at 1 or 3 months of age (data not shown). Thus, the breakdown of the filtration barrier was not due to simple podocyte depletion at these early times. However by 12 months of age, the extensive network of Nephrin staining was partially characterized.

Glomerular interdigitation of cellular processes [35]. Given that podocyte foot processes are also actin based and require some type of guidance, we examined the role of Ntrk3 further. Quantitative RT-PCR confirmed that Ntrk3 expression was down approximately 10 fold in glomerular preps from PTIP− compared to PTIP+ animals (Figure 5A). We also examined Ntrk3 levels in kidneys by co-immunostaining kidney sections with Ntrk3, WT1 and Nephrin antibodies (Figure 6). At 3 months of age, Ntrk3 could be seen in glomeruli of PTIP+ kidneys, however the staining intensity in PTIP− kidneys was severely reduced in almost every glomerulus examined (Figure 6D, 6J). Some slight filamentous staining

Alteration of the Gene Expression Program Precedes the Disease Phenotype

Alterations in cellular phenotypes could be the result of changes in the transcriptional program of PTIP− podocytes. Thus, we prepared RNA from glomeruli enriched fractions at 1 month of age, prior to the onset of any significant phenotype, and assayed for gene expression changes by Affymetrix microarrays. We compared glomerular RNA preps from 10 independent PTIP− animals and 8 PTIP+ littermates at 1 month of age. The data were highly consistent and indicated both gain and loss of gene expression in the PTIP− kidneys (Table 1 and Table 2). The entire dataset can be accessed at the Gene expression Omnibus (GSE17709). Expression changes were confirmed by quantitative RT-PCR for selected genes (Figure 5). Among the genes increased was Protamine 1 (Ptn1), which is not normally expressed in podocytes or other somatic cells but is found only in spermatids where it is essential for chromatin condensation and fertility [30,31]. The changes in RNA expression observed were surprising and did not correspond to any common pathways. In fact, the podocyte-specific genes that are known to function in cell viability and slit diaphragm integrity were largely unchanged (Table S1 and Figure 5C). The data suggest that loss of PTIP in podocytes alters the transcriptional program to affect a limited number of genes whose functions in the podocytes have not been previously characterized.

PTIP Deletion Affects Ntrk3 Expression and Histone Methylation

Among the most interesting genes whose expression was down regulated in PTIP− kidneys was the neurotrophic tyrosine kinase receptor type 3 (Ntrk3, formerly called TrkC), whose expression in podocytes had not been previously described. The Ntrk3 gene encodes two proteins that recognize neurotrophin 3 (NT-3) and functions in axon guidance and innervation and in cardiac development [32–34]. Ntrk3 promotes axon outgrowth and guidance, presumably through actin based extension and retraction of cellular processes [35]. Given that podocyte foot processes are also actin based and may require some type of guidance, we examined the role of Ntrk3 further. Quantitative RT-PCR confirmed that Ntrk3 expression was down approximately 10 fold in glomerular preps from PTIP− compared to PTIP+ animals (Figure 5A). We also examined Ntrk3 levels in kidneys by co-immunostaining kidney sections with Ntrk3, WT1 and Nephrin antibodies (Figure 6). At 3 months of age, Ntrk3 could be seen in glomeruli of PTIP+ kidneys, however the staining intensity in PTIP− kidneys was severely reduced in almost every glomerulus examined (Figure 6D, 6J). Some slight filamentous staining
remained in the PTIP− glomeruli, but the overall intensity was markedly different. In PTIP+ glomeruli, Ntrk3 staining was remarkably similar to Nephrin (Figure 6G–6I). However, Nephrin staining intensity was unaffected in PTIP− glomeruli even though Ntrk3 was much lower (Figure 6J–6L). The Nbk3 expression in glomerular preps and its decrease in the PTIP− kidneys suggested a function in foot process growth, guidance, and/or pattern formation.

In order to more directly link PTIP to the Nbk3 locus, we designed chromatin immunoprecipitation experiments to examine the presence of PTIP and the changes in histone methylation patterns around the transcription initiation site (+1) of Nbk3 (Figure 7). Chromatin was prepared from whole glomerular preps from PTIP+ and PTIP− kidneys, which also included mesangial and endothelial cells. Despite the presence of other cell types in the glomerular chromatin, we were able to detect a 5–6 fold decrease in PTIP localization to sequences around the start site of Nbk3 transcription when comparing PTIP+ to PTIP− chromatin (Figure 7B). No significant amount of PTIP was detected further upstream (~1200), nor did we see a significant difference, between PTIP+ and PTIP− chromatin, in PTIP localization within the 5′ UTR of exon 1 (Figure 7B, P4 site). Clear differences in H3K4me2 were also measured, with an approximately 50–60% decrease in PTIP− chromatin with primer pairs P2–P4, but not with P1 at ~1200 (Figure 7C). Similarly, H3K4me3 levels were also decreased in PTIP− chromatin at P2–P4 but not at P1 (Figure 7D). We also examined changes in Polycomb mediated epigenetic silencing marks using an antibody against H3K27me3 (Figure 7E), which appeared unchanged at all sites examined. These data demonstrate recruitment of PTIP to the promoter region of Nbk3 in normal glomeruli.

Ntrk3 Mutants Have Podocyte Foot Process Defects

In order to determine if the loss of Ntrk3 alone would impact normal glomerular patterning, we examined homozygous Nbk3 mutant mice. The Nbk3 mutants die shortly after birth due to cardiac and neuromuscular defects; however their kidneys had not been studied previously. Therefore, we collected urine and kidney tissue for light and electron microscopy from 3–4 day old Nbk3 mutants and littersmates. At three days post partum, Nbk3 mutants were small and sickly. Higher levels of albumin could be observed in the urines of Nbk3−/− pups (Figure 8A), compared to control littersmates, although this could be due to delayed or arrested kidney development. Glomerular development was examined in kidney sections of 4 day old newborns (Figure 8B). At this time, nephrins are still undergoing development and glomeruli at the periphery are just beginning to form whereas cortical glomeruli closer to the medulla are already fully functional. The tight junction protein Magi2 specifically localizes to podocyte cell junctions and exhibited altered patterning in Ntrk3 mutant kidneys, with discontinuous staining and excessive looping of the developing tuft. In mature glomeruli, Nephrin staining was reduced and patchy in the Nbk3 mutants. The number of podocytes did not seem affected in the Nbk3−/− mice at this time.

Ultra structural analysis of Ntrk3 mutant kidneys revealed podocyte patterning defects both by scanning and transmission EM (Figure 9). At 4 days post-partum, we examined the most mature glomeruli, those located closest to the medullary zone. Podocyte foot processes from Ntrk3−/− mice exhibited disorganized secondary and tertiary processes that crisscrossed randomly over capillary vessels and were poorly interdigitated (Figure 9A, 9B). Few sections showed the characteristic spacing indicative of the slit diaphragms at the glomerular basement membranes (Figure 9D). These data suggest a critical role for Ntrk3 in the fine patterning events of secondary and tertiary foot process formation and interdigitation.

Discussion

In this report, we utilized a conditional deletion to ask whether the PTIP dependent H3K4 methylation function is required in a terminally differentiated cell type, to maintain its differentiated state and its cell-type specific transcriptional program. Using the glomerular podocyte cell as a model, we show that deletion of PTIP results in subtle changes in gene expression patterns that ultimately lead to a slowly progressing disease state. These data support a model in which the gross stability of the differentiated state or podocyte cell survival, at least in the short term, does not depend on the PTIP/KMT2 complex, as many of the podocyte specific genes examined were unchanged in the absence of PTIP. Rather, the loss of PTIP was more subtle and revealed unexpected changes in a small number of genes and ultimately led to a chronic disease phenotype resembling glomerular sclerosis. Typical characteristics of chronic glomerular disease were present, including microalbuminuria, podocyte foot process fusion or effacement, remodeling of the filtration barrier, and increased extracellular matrix deposition.

Methylation of histone H3 at lysine 4 correlates with gene expression and is thought to regulate cellular identity by establishing and maintaining a stable epigenetic state. The PTIP protein is part of an H3K4 methyltransferase complex that includes the mammalian Trithorax homologues KMT2B and/or KMT2C [10,11,13,16]. Previous studies in flies and mice demonstrated reduced H3K4 methylation in Paxip1 mutants and severe early lethal phenotypes. In the mouse, complete loss of PTIP protein results in developmental arrest just after gastrulation [14], a phenotype more severe than any individual mouse KMT2 family gene mutation [12,36,37], whereas a hypomorphic Paxip1 allele is lethal later in development [38]. In flies, maternal and zygotic ptip null embryos are embryonic lethal and fail to express many segmentation genes [17]. In mouse embryonic stem cells, PTIP protein is required for normal levels of H3K4 methylation and for maintaining pluripotency in cell culture [15], whereas in embryonic fibroblasts PTIP is required for adipocyte differentiation [16]. All of these findings suggest that a PTIP H3K4 methyltransferase complex is needed for differentiation of stem cells and progenitor cells in development. However in terminally differentiated cells, the requirement for active H3K4 methylation may be different and the lack of cell division may abrogate the need for de novo methylation. Our results suggest that PTIP must still function in some non-dividing cells, perhaps as part of a maintenance complex, as overall levels of H3K4 methylation were reduced and activation and suppression of a small number of genes was affected.
Figure 3. Podocyte Viability and Glomerular Morphology. A) After immunostaining with WT1 and Nephrin antibodies, podocyte nuclei were counted in mid-cross sections through glomeruli whose vascular and proximal tubular poles were visible. Glomerular surface area for mid-cross sections was measured by morphometry and is expressed in relative units. B) Immunostaining for WT1 (pink) and Nephrin (green) at 3 months of age shows little significant difference between PTIP+ and PTIP- glomeruli. However, Podocin staining (green, lower panels) appears less and discontinuous in PTIP- glomeruli. Nuclei were counterstained with DAPI. By 12 months, large regions cleared of Nephrin positive staining were evident within the glomerular tufts of PTIP- animals.
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The mature podocyte is generally believed to be a non-dividing cell type, as classic cell BrdU labeling experiments do not mark this population over time [39]. However, more recent genetic lineage tracing experiments suggest that there is a population of parietal epithelial cells at the vascular pole of the Bowman’s capsule that can replenish podocytes over time [40,41]. This replacement of podocytes appears slow under normal conditions, but may be especially critical in cases of glomerular injury. In our animal model, we would expect any podocyte replacement to also delete the Paxip1 gene once expression of the Cre driver is activated.

Given that we do not see significant loss of podocytes until at least 6 months of age, it may be that alterations in the transcriptional profile are not lethal. Rather, loss of podocytes may be the result of the damaged filtration barrier, the increase in the mesangium, and the general environment of the glomerular injury. In our animal model, we would expect any podocyte replacement to also delete the Paxip1 gene once expression of the Cre driver is activated. Given that we do not see significant loss of podocytes until at least 6 months of age, it may be that alterations in the transcriptional profile are not lethal. Rather, loss of podocytes may be the result of the damaged filtration barrier, the increase in the mesangium, and the general environment of the glomerulus in older mice. Alternatively, if podocyte replacement is accelerated in our model, it may be that by 6 months the ability of parietal cells to replenish the podocyte population is exhausted. In either case, the effects of manipulating the H3K4 methylation pathway is more apparent in older mice, suggesting a critical role for such epigenetic pathways in aging cells and tissues.

The changes in gene expression observed in response to PTIP deletion are surprising in that most of the well-characterized podocyte-specific genes appear unaffected. However, changes include both activation and suppression of previously uncharacterized genes in the podocytes. Activation of the Prm1 gene in PTIP−/− kidneys is unusual as this gene has only been associated with sperm maturation and is thought to encode a unique chromatin binding protein [31,42]. Activation of the Padi4 gene could impact gene expression by deimination of arginines in the histone H3 tail, which prevents methylation [43]. The impact of increased Padi4 is likely to be complex as arginine methylation can correlate with gene activation or repression, depending on the context and specific residues.

The most compelling gene affected in PTIP−/− podocytes was Ntrk3, whose expression in the glomerulus had not been previously characterized. The reduction of Ntrk3 expression in PTIP−/− kidneys and the phenotype of Ntrk3−/− newborn kidneys suggest that this receptor is critical for tertiary foot process pattern formation. The podocyte is a highly specialized cell with a complex network of processes that cover the glomerular basement membrane. The large primary processes are microtubule containing structures, whereas the tertiary, interdigitated foot processes contain actin microfilaments [44]. Adjacent foot processes are connected through a specialized junctional complex, called the slit diaphragm, which is essential for maintaining a functional filtration pore. Some of the essential proteins in the slit-diaphragm, such as Nephrin, Podocin, and Neph1 are well characterized and

Figure 4. Ultrastructural Analysis of PTIP−/− Kidneys. Podocytes of PTIP−/− mice showed progressive foot process disorganization and effacement, as observed by scanning (A–C, G, H) and transmission (D–F, I, J) electron microscopy. Podocyte foot processes of 3-month-old PTIP−/− mice were regularly interdigitated (A, D, G), whereas those of age-matched PTIP−/+ podocytes (B, C, E, F, H) displayed varying degrees of disorganization (B, E) and effacement (C, F). Note that slit diaphragms could still be observed between foot processes during the early stages of disorganization (E, arrows). G–J) In addition to the foot process alterations, capillary loop deformation/enlargement (H, J) and mesangium expansion (J, asterisks) were observed in glomeruli of 12-month-old (G, H) and 3-month-old (I, J) mice analyzed by EM. Scale bars: (A–C) 1 μm; (D–F) 100 nm; (G–J) 2 μm.

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### Table 1. Genes Up-Regulated in PTIP—Podocyte.

| Probe     | Symbol   | Description                          | UniGene  | p-value | Fold Change* |
|-----------|----------|--------------------------------------|----------|---------|--------------|
| 1439379   | Prm1     | protamine 1                          | Mm.42733 | 0       | 6.38         |
| 1418398   | Tspan32  | tetraspanin 32                       | Mm.28172 | 0       | 2.92         |
| 1422760   | Padi4    | peptidyl arginine deiminase, type IV | Mm.250358| 0.001   | 1.90         |
| 1433744   | Lrm2     | leucine-rich repeats and transmembrane domains 2 | Mm.121498| 0       | 1.89         |
| 1435259   | E430002G05Rik | RIKEN cDNA E430002G05 gene     | Mm.28649 | 0       | 1.77         |
| 1436329   | Egr3     | early growth response 3              | Mm.103737| 0       | 1.74         |
| 1449071   | Myl7     | myosin, light polypeptide 7, regulatory | Mm.46514 | 0.001   | 1.68         |
| 1419527   | Comp     | cartilage oligomeric matrix protein  | Mm.45071 | 0       | 1.58         |
| 1419487   | Mybph    | myosin binding protein H             | Mm.379067| 0.001   | 1.30         |
| 1431991   |         | 2410004P03Rik RIKEN cDNA 2410004P03 gene | Mm.227543| 0       | 1.26         |
| 1430062   | Hhip1    | hedgehog interacting protein-like 1  | Mm.6423  | 0.004   | 1.19         |
| 1453228   | Stx11    | syntaxin 11                          | Mm.248648| 0.003   | 1.16         |
| 1416077   | Adrnmedullin | adrenomedullin                     | Mm.1408  | 0       | 1.14         |
| 1457780   | Stx11    | syntaxin 11                          | Mm.248648| 0.001   | 1.10         |
| 1434984   |         | 6330514A18Rik RIKEN cDNA 6330514A18 gene | Mm.17613 | 0.004   | 1.08         |
| 1453152   | Mamdc2   | MAM domain containing 2              | Mm.50841 | 0.012   | 1.05         |
| 1445098   |         | 3110006E14Rik RIKEN cDNA 3110006E14 gene | Mm.227543| 0.004   | 1.00         |
| 1453830   |         | 5430435G22Rik RIKEN cDNA 5430435G22 gene | Mm.44508 | 0.002   | 1.01         |
| 1439761   |         | D830026I12Rik RIKEN cDNA D830026I12 gene | Mm.136046| 0.008   | 1.00         |

* log2 scale.  
doi:10.1371/journal.pgen.1001142.t001

### Table 2. Genes Down-Regulated in PTIP—Podocytes.

| Probe     | Symbol   | Description                          | UniGene  | p-value | Fold Change* |
|-----------|----------|--------------------------------------|----------|---------|--------------|
| 1425425   | Wif1     | Wnt inhibitory factor 1              | Mm.32831 | 0       | -4.37        |
| 1441491   | A330068G13Rik | RIKEN cDNA A330068G13 gene   | Mm.227543| 0       | -3.68        |
| 1433825   | Ntrk3    | neurotrophic tyrosine kinase, receptor, type 3 | Mm.33496 | 0       | -3.09        |
| 1446222   | A330068G13Rik | RIKEN cDNA A330068G13 gene   | Mm.227543| 0       | -2.14        |
| 1452779   | 3110006E14Rik | RIKEN cDNA 3110006E14 gene | Mm.23960 | 0       | -1.57        |
| 1452416   | Il6ra    | interleukin 6 receptor, alpha        | Mm.2856  | 0       | -1.55        |
| 1420903   | St5galnac3 | stitactin 2                         | Mm.44029 | 0       | -1.53        |
| 1450309   | Atn2     | astrotactin 2                        | Mm.445312| 0       | -1.53        |
| 1433939   | Af7f     | A7f/FMRFamide family, member 3       | Mm.336679| 0       | -1.53        |
| 1437403   | Samd5    | sterile alpha motif domain containing 5 | Mm.101115| 0.001   | -1.48        |
| 1429996   | S830408B19Rik | RIKEN cDNA S830408B19 gene | Mm.291322| 0       | -1.35        |
| 1455296   | Adcy5    | adenylyl cyclase                     | Mm.41137 | 0       | -1.30        |
| 1431946   | Necab3   | N-terminal EF-hand calcium binding protein 3 | Mm.143748| 0       | -1.29        |
| 1447777   | Mym1     | v-myc myelocytomatosis viral oncogene homolog 1 | Mm.1055  | 0       | -1.26        |
| 1419139   | Gdf5     | growth differentiation factor 5      | Mm.4744  | 0.001   | -1.25        |
| 1441559   | LOC627626 | similar to CG11212-PA                | Mm.390999| 0.003   | -1.25        |
| 1441667   | Smyd1    | SET and MYND domain containing 1     | Mm.234274| 0       | -1.23        |
| 1423561   | Neil2    | NEL-like 2 (chicken)                 | Mm.3959  | 0.016   | -1.18        |
| 1450501   | Itga2    | integrin alpha 2                     | Mm.5007  | 0       | -1.17        |
| 1435832   | Lrrc4    | leucine rich repeat containing 4     | Mm.443660| 0       | -1.11        |
| 1455188   | Ephb1    | Eph receptor B1                      | Mm.22897 | 0.046   | -1.11        |
| 1455888   | Lingo2   | leucine rich repeat and Ig domain containing 2 | Mm.132507| 0.007   | -1.05        |
| 1462960   | Fa2h     | fatty acid 2-hydroxylase             | Mm.41083 | 0       | -1.04        |
| 1453841   | 2310050P20Rik | RIKEN cDNA 2310050P20 gene | Mm.44508 | 0.033   | -1.01        |
| 1421207   | Lif      | leukemia inhibitory factor           | Mm.4964  | 0       | -1.00        |

* log2 scale.  
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cultural neuronal cells, NT-3 promotes localization of 
actin polymerization and lamellipodia formation [46,47]. In 
NT-3 is known to promote neuronal axon guidance by stimulating 
NT–3, may be important for foot process growth and patterning. 
Yet, how foot process outgrowth is regulated and maintained is not 
clear. Our data suggests that Ntrk3, and by inference its ligand 
mutations are associated with severe nephrotic syndromes [45]. 
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mutations are associated with severe nephrotic syndromes [45]. 

Figure 5. Gene Expression in the Glomerulus. Real-time qRT-PCR 
for the indicated genes was performed on total RNA isolated from 
glomerular preparations. A) Confirmation of two genes that are down-
regulated in PTIP− (black) kidneys compared to controls PTIP+ (open) 
kidneys. B) Confirmation of two genes that are up-regulated in PTIP− 
kidneys compared to controls. C) Expression levels of podocyte marker 
genes in PTIP+ and PTIP− glomerular preparations. 
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other genes whose functions are not well understood are also 
impacted.

Histone methylation by Trithorax or Polycomb complexes can 
imprint positive and negative epigenetic marks on chromatin 
during development. More recently, histone methyltransferases 
have been associated with cancer and other disease states. 
However, in many cases it is not clear whether changes in the 
expression of epigenetic modifiers are the cause or the result of 
disease progression. The results presented here suggest that 
mutations in an epigenetic pathway, which result in alterations of 
H3K4 methylation patterns, can lead to a chronic disease 
through subtle changes in gene expression patterns. This implies a 
direct function for HMTs in maintaining gene expression and the 
differentiated state in healthy organisms.

Methods

Animals

Mice carrying the Paxip1 null (Paxip1−/−) and floxed [Paxip1fl] 
alleles were previously described and genotyped as indicated 
[14,53]. To obtain the specific deletion of the Paxip1fl allele in 
glomerular podocytes, these mice were crossed with the previously 
characterized 2.5P-Cre mice [24,25], which express the Cre 
recombinase under the control of the human NPHS2 promoter 
(CreNPHS2). Among the next generations, mice carrying the Cre 
allele (Paxip1fl/fl;CreNPHS2 and Paxip1fl/fl:CreNPHS2 mice) were 
considered as conditional null mutants (PTIP−), whereas 
littermates that did not express the Cre recombinase were used 
as controls (PTIP+). All animal procedures were approved by the 
University Committee on Use and Care of Animals (UCUCA) of 
the University of Michigan and performed in compliance with 
ULAM recommendations.

Antibodies

Rabbit polyclonal antibodies used to detect Nephrin (1:1000) and 
Podocin (1:500) were kindly provided by L.B. Holzman 
(University of Pennsylvania, Philadelphia, PA). Chicken anti-PTIP 
was described previously [54]. Additional antibodies were 
commercially available: mouse clone 6F-H2 anti-WT1 (1:1000, 
DAKO, Carpinteria, CA), anti-H3K4me3 and anti-H3K27me3 
(University of Pennsylvania, Philadelphia, PA). Chicken anti-PTIP 
was described previously [54]. Additional antibodies were 
commercially available: mouse clone 6F-H2 anti-WT1 (1:1000, 
DAKO, Carpinteria, CA), anti-H3K4me3 and anti-H3K27me3 
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(University of Pennsylvania, Philadelphia, PA). Chicken anti-PTIP
Figure 6. Ntrk3 in the Glomerulus. Fresh frozen tissues were sectioned and fixed in methanol followed by immunostaining with goat anti-Ntrk3, rabbit anti-WT1, or rabbit anti-Nephrin, as indicated. PTIP+ sections (A–C, G–I) showed strong Ntrk3 staining in all glomeruli, in a pattern similar to Nephrin. The PTIP− kidney sections (D–F, J–L) showed much lower levels of Ntrk3 protein in glomeruli. All micrographs were taken at manually set, equal exposures. Right panels (C, F, I, L) are overlays of Ntrk3 and WT1 or Ntrk3 and Nephrin and are counterstained with DAPI (blue) to visualize all cell nuclei.

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ptertobarbital and prepared for systemic perfusion. A saline solution was first injected through the abdominal aorta to the entire mouse body at a pressure of approximately 70 mmHg as previously described [55]. As soon as the general bloodstream had been cleared, a solution of 4% paraformaldehyde in PBS was substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substituted. It was left to perfuse at the same flow conditions for at least 10 minutes. Kidneys were removed, decapsulated, substitute
which concentration and purity were determined by nanodrop analysis on an Agilent Bioanalyzer 2100 (Agilent Technologies).

Microarray and Real-Time qPCR Analyses

Microarray analyses were done by the University of Michigan Comprehensive Cancer Center (UMCCC) Affymetrix and Microarray Core Facility. The FL-Ovation cDNA Biotin Module V2 kit (NuGEN Technologies, San Carlos, CA) was used to produce biotin-labeled cRNA, which was then fragmented and hybridized to a Mouse 430 2.0 Affymetrix GeneChip 3 expression array (Affymetrix, Santa Clara, CA). Array hybridization, washes, staining, and scanning procedures were carried out according to standard Affymetrix protocols. Expression data were normalized by the robust multiarray average (RMA) method and fitted to weighted linear models in R, using the affy and limma packages of Bioconductor, respectively [59,60]. Only probe sets with a variance over all samples superior to 0.1, a p-value inferior or equal to 0.05 after adjustment for multiplicity using the false discovery rate [61], and a minimum 2-fold difference in expression were selected for the analysis. The complete data set is available from the Gene Expression Omnibus database (accession number GSE17709).

Microarray data were confirmed by real-time quantitative PCR analysis. 25–50 ng single-stranded cDNA was amplified in triplicate in a 384-well plate, using the 7900HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA) and expression levels of selected genes was determined by SYBR Green or TaqMan assays (Applied Biosystems). PCR primers pairs and TaqMan probes used in this study are presented in Table S2.

Chromatin Immunoprecipitation

Glomeruli were isolated from 6 PTIP+ and 6 PTIP− kidneys by sieving as described above. Glomeruli were resuspended in 1 ml PBS and cross linked with 1% formaldehyde for 10 minutes with rocking at room temperature, Chromatin preparation, immunoprecipitation, and PCR analysis was essentially as described previously [13]. Primers pairs for the Ntrk3 locus were as follows: P1, 5’-CAATGTATTTGCTTCCTGCC, 5’-AAGAAAGG-
GTAGGGGACTCCG; P2, 5'- AACCCTGCGTCTTGG- 
TAAGG, 5'- GGAGGAGGAGGAGGAGGAG- 
P3, 5'- GCATCTTCTTCCTCCTCCTCC- 
TAAGG, 5'- AAGTCACAAAGTCACCTCAG- 
P4, 5'- TTGGCCTCCACCGTC-TGTGG, 5'- TGGCTTIGAAGGCAGCAGAC.

Supporting Information
Table S1 Podocyte-specific genes that are unchanged after PTIP deletion.
Found at: doi:10.1371/journal.pgen.1001142.s001 (0.03 MB DOC)

Table S2 Quantitative RT-PCR primer sets and probes.
Found at: doi:10.1371/journal.pgen.1001142.s002 (0.02 MB DOC)

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