A WT1 Co-regulator Controls Podocyte Phenotype by Shuttling between Adhesion Structures and Nucleus*

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Glomerular podocyte differentiation state is critical for filtration barrier function and is regulated by WT1, a zinc finger transcription factor. A yeast two-hybrid assay identified a novel, WT1-interacting protein (WTIP) that maps to human chromosome 19q13.1, a region with genes linked to familial focal segmental glomerulosclerosis. The domain structure of WTIP is similar to the zyxin subfamily of cytosolic LIM domain-containing proteins, which contain three carboxyl-terminal LIM protein-protein interaction domains and a proline-rich, pre-LIM region with a nuclear export signal. Other LIM domain-containing proteins (zyxin and mouse muscle LIM protein) did not interact with WT1 in two-hybrid assays, and WTIP did not interact with an unrelated transcription factor, LMX1B. WTIP mRNA was detected in cultured podocytes and was developmentally regulated, with expression peaking in mouse kidney at embryonic day 15–16 (E15–E16) in kidney but persisting into adulthood. In situ hybridization demonstrated WTIP expression in developing E15 glomeruli and in cultured podocytes. The partial WTIP clone, which interacted with WTIP in the two-hybrid assay, co-localized with WT1 in nuclei, co-precipitated with WT1, and inhibited WT1-dependent transcriptional activation of the amphiuregulin promoter. In contrast, full-length WTIP was excluded from cell nuclei, but after the addition of leptomycin B, an inhibitor of Crm1-mediated nuclear export, it accumulated in the nucleus and co-precipitated with WT1 in whole cell lysates. Epitope-tagged WTIP co-localized with the adaptor protein CD2AP (CMS) in podocyte actin spots and with Mena at cell-cell junctions. We propose that WTIP monitors slit diaphragm protein assembly as part of a multiple protein complex, linking this specialized adhesion junction to the actin cytoskeleton, and shuttles into the nucleus after podocyte injury, providing a mechanism whereby changes in slit diaphragm structure modulate gene expression.

Podocytes are highly specialized epithelial cells, which synthesize components of glomerular basement membrane, elabotate interdigitating foot processes from adjacent cells that encircle capillaries and are bridged by extracellular proteins of the slit diaphragm. In proteinuric renal diseases, podocytes undergo stereotypic phenotypic simplification into a cuboidal shape, characterized by foot process fusion and retraction and loss of filtration barrier function. Although persistent podocyte dysregulation is associated with glomerular scarring, this phenotype switch is reversible. Appropriate therapy can restore normal podocyte structure and function, suggesting a dynamic, regulated process. Positional cloning and gene targeting have identified the proteins critical for normal podocyte function. However, in the absence of mutations, molecular mechanisms that regulate podocyte phenotype remain unclear. Given its unique microenvironment with exposure to hemodynamic forces and high flow of ultrafiltrate, we speculated that podocytes express intracellular molecules that relay changes in extracellular physical forces or soluble signals to modify cellular structure and function.

A number of podocyte proteins are candidate targets as putative regulatory molecules. Normal podocyte differentiation requires the correct temporal expression of specific transcription factor genes (1). Of this gene set, we focused on the zinc finger transcription factor WT1 for several reasons. Although originally discovered as a tumor suppressor gene inactivated in a subset of Wilms’s tumors, WT1 is essential for normal nephrogenesis. WT1 null mice lack kidneys (2), and, when engineered to express a human WT1 transgene, the animals survive but develop mesangial sclerosis or crescentic nephritis (3), depending on the WT1 expression level. In normal adult animals, WT1 expression is restricted to podocytes, suggesting a role for WT1 in maintenance of podocyte differentiation. In fact, WT1 zinc finger mutations are associated with the re-expression of an immature podocyte phenotype (4) and Denys-Drash and Frasier syndromes (5), rare causes of familial glomerulosclerosis. Mice engineered to express these WT1 mutations develop phenotypes that parallel the human syndromes (5–7), further documenting that WT1 function is required for a normal podocyte phenotype. Finally, WT1 expression levels decrease in acquired causes of glomerulosclerosis (8, 9).

The WT1 gene encodes at least 24 different isoforms, with the four major isoforms represented by two alternative splice sites (5, 10–12). The first alternative splice site either includes or excludes 17 amino acids encoded by exon 5 and is only expressed in mammals. The function of this domain is not clear, since mice engineered to express WT1 isoforms without...
exon 5 develop normally and are fertile (13). The second alternative splice site occurs in intron 9 and includes or excludes three amino acids, KTS. In vitro, WT1 isoforms without KTS (WT1 lacking KTS) bind DNA and regulate transcription, whereas those with the KTS (WT1 including KTS) insert primarily regulate RNA processing but not gene activity (5). WT1 is both a transcriptional repressor and activator (11) and regulates genes important in nephron formation including podocalyxin (14), amelogenesis (15) and perhaps nephrin (3).

To understand mechanisms of podocyte differentiation more completely, we identified proteins that interacted with WT1, using a yeast two-hybrid system with a full-length WT1 isoform containing exon 5 and lacking the KTS as bait (WT1+/-). We anticipated finding a nuclear cofactor that regulated WT1 activity. Instead, we cloned a novel, cytosolic molecule, WT1-interacting protein (WTIP), with a domain structure similar to the zyxin family of LIM domain-containing scaffolding proteins. In vitro functional studies demonstrated that when sequestered within the nucleus, WTIP repressed RNA processing but not gene activity (5). WT1 is both a transcriptional repressor and activator (11) and regulates genes important in nephron formation including podocalyxin (14), amelogenesis (15) and perhaps nephrin (3).

In Situ Hybridization—Nonradioactive in situ hybridization was performed on 5-μm paraffin sections from microdissected E15–16 kidneys, which were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned as described (18). To generate riboprobe templates, a mouse 800-bp WTIP cDNA fragment was subcloned bidirectionally into pCRII. DIGoxigenin-labeled, nonhybridizing and hybridizing RNA probes were synthesized using T7 RNA polymerase (Roche Applied Science) and were quantified spectrophotometrically and by dot blot. In situ hybridizations were performed using equivalent concentrations of sense and antisense probe. Hybridized probe was detected using an anti-DIGoxigenin antibody. Prehybridization, hybridization, and washes were performed at 50 °C.

Generation of Expression Vectors and Transient Transfections—Expression vectors for full-length WTIP, WTIP with deletion of the LIM domain (N-ΔLIM) and WTIP full length (N+LIM) were generated by cloning the coding regions into pcCMV-Tag (Stratagene, La Jolla, CA) with fusion to an N-terminal FLAG or Myc tag to generate pcCMV-Tag-FLAGWT1 (ΔLIM), pcCMV-Tag-Myc-NΔLIM, and pcCMV-Tag-MycWTIP. pcDNA3-HA-CD2AP was a gift from Dr. Larry Holzman (University of Michigan). COS7 cells, 3T3 fibroblasts, or podocytes were transiently transfected with the indicated expression vector using Superfect (Qiagen) according to the manufacturer’s protocol. Briefly, cells were cultured for 6 h with 5 μg of pcDNA-ΔLIM or pcDNA-NΔLIM in transfection medium containing 10% fetal bovine serum. Cells were either harvested 48 h after transfection for biochemical analyses or incubated in 4% paraformaldehyde in PBS for immunocytochemical studies.

Immunofluorescence—Transiently transfected cells cultured on sterile glass coverslips (collagen type I-coated for podocytes) were washed in Dulbecco’s PBS, fixed in paraformaldehyde (4%, 20 min, 4 °C), and permeabilized with 0.2% Triton X-100 in Dulbecco’s PBS for 30 min at room temperature. After blocking with 10% goat serum, cells were incubated with primary antibodies in 0.2% Triton-X 100, 0.1% gelatin in PBS for 2 h at room temperature. Secondary antibodies were fluorescein isothiocyanate-conjugated, TRITC-conjugated, and FITC-conjugated, and antibodies were reference conjugated to goat anti-rabbit (Jackson ImmunoResearch Laboratories) on standard class slides. Anti-Mena antibody was a gift from B. Wang (Case School of Medicine). Subsequently, coverslips were washed and incubated with secondary antibody at dilutions ranging from 1:200 to 1:300 for 1.5 h at room temperature. Secondary antibodies were fluorescein isothiocyanate-conjugated horse anti-mouse or Texas Red-conjugated goat anti-rabbit antibodies (Vector Laboratories, Burlingame, CA). Coverslips were mounted in anti-fade, aqueous medium containing 4’,6-diamidino-2-phenylindole (Vectashield, Vector Laboratories) on standard glass slides. Anti-Mena antibody was a gift from B. Wang (Case School of Medicine). Subsequently, coverslips were washed and incubated with secondary antibody at dilutions ranging from 1:200 to 1:300 for 1.5 h at room temperature. Secondary antibodies were fluorescein isothiocyanate-conjugated horse anti-mouse or Texas Red-conjugated goat anti-rabbit antibodies (Vector Laboratories, Burlingame, CA). Coverslips were mounted in anti-fade, aqueous medium containing 4’,6-diamidino-2-phenylindole (Vectashield, Vector Laboratories) on standard glass slides. Anti-Mena antibody stained the nuclei of all cells in the examined area. Confocal images were obtained with a Leica TCS SP2 confocal system. Digital images were processed and grouped using Adobe Photoshop v6.0 (Adobe Systems Inc., San Jose, CA).
A Novel WT1 Co-regulator

RESULTS

Methods

WTIP Domain Structure

WTIP is required for appropriate podocyte differentiation, but mechanisms regulating its activity and expression are incompletely understood. To identify proteins that interact with WT1, we used a yeast two-hybrid assay to screen a cDNA kidney library from adult mice with a full-length WT1 isoform containing exon 5 and lacking the KTS insertion after exon 9 (WT1(−KTS)) as bait. A total of 1.2 × 10^6 transformants were screened, yielding 18 positive yeast colonies. Two plasmids encoded identical 1.2-kb nt expressed sequence tag sequences (AA050259 and AI232654), and a partial amino acid sequence was deduced by translating the expressed sequence tag nucleotide sequence in frame with the GAL4 activation domain. Two-hybrid assays demonstrated that WTIP and WT1 specifically interact. This partial WTIP sequence did not self-activate or interact with a negative control yeast GAL4 binding domain fusion protein or irrelevant transcription factor LMX1B (Table I).

Full-length mouse and human WTIP cDNA sequences were generated using information in the NCBI and Celera databases. The mouse, partial WTIP cDNA identified in the two-hybrid assay mapped to chromosome 7 and was orthologous to a human gene on chromosome 19q13.12, which contained additional in-frame sequence with an initiation codon. BLASTn searches identified new human and mouse expressed sequence tags, which were obtained from the IMAGE consortium and sequenced. The resulting cDNA sequence overlapped with the expressed sequence tag sequence identified in the yeast two-hybrid assay and allowed construction of clones containing full-length 430-amino-acid human and 398-amino-acid mouse coding sequences for a novel WTIP. Alignment of the full-length mouse and human WTIP protein sequences is shown using ClustalW in Fig. 1. Overall, the proteins are 89% identical and 94% conserved. The primary sequence divergence between mouse and human WTIP proteins is restricted to the amino terminus. Human WTIP contains two short amino acid stretches and a polyglycine repeat that are not present in mouse WTIP, suggesting these residues are not critical for WTIP function.

The Domain Organization of WTIP Is Similar to Other Zyxin Family Paralogues—Analysis of both mouse and human WTIP using SMART (available on the World Wide Web at smartanelled-heidelberg.de) identified three carboxy-terminal LIM protein-protein interaction domains, which demonstrated significant alignment with the zyxin family of LIM-domain containing proteins. Yeast co-transfected with the GAL4 binding domain-WTIP fusion protein expression plasmid and plasmids encoding fusion proteins containing GAL4 activation domain and other LIM domain-containing proteins (mouse muscle LIM protein (also known as cysteine-rich protein) or the LIM domains of zyxin, which localize to the nucleus. c Mouse muscle LIM protein (orthologous with human cysteine-rich protein).

WTIP specifically interacts with WT1 in yeast

| GALA AD† fusion protein | GALA BD† fusion protein | Reporter gene |
|-------------------------|-------------------------|---------------|
| WTIP (LIM only)†a | WT1(−KTS) | + + | Blue |
| GALA BD | GALA BD | White |
| Lamin | Lamin | White |
| LMXI B | LMXI B | White |
| WT1(−KTS) | WT1(−KTS) | + + | Blue |
| MMLP†b | WT1(−KTS) | − | White |
| Zyxin (LIM only)†c | WT1(−KTS) | − | White |
| Lamin | Lamin | White |
| GALA AD | GALA AD | White |

†a Activation domain.
†b Binding domain.
†c Fusion proteins contain only the LIM domain regions of WTIP and zyxin, which localize to the nucleus.

WTIP is a novel LIM-only parologue with a unique domain structure containing a unique zinc-finger insertion and divergent LIM domains. The unique insert permits the WTIP family to be categorized separately from other members and to have unique functional specificity in this family of proteins through unique protein-protein interactions.
are identical on average with the other zyxin family members, and no stretches of amino acids encoding motifs are conserved. Human and mouse WTIP contain two conserved, proline-rich regions that appear to be SH3 binding domains (Pro151 (AQF-PFPLPSLPLPP) and Pro 157 (LPSLPLPPGREGGPS) in mouse WTIP and Pro 183 (GPAPFPLPALPLPPG) and Pro 189 (LPALPLPLPPGREGGPS) in human WTIP). Both mouse and human WTIP contain a PDZ binding domain (VTEL) at the carboxyl terminus (Fig. 2), a motif that distinguishes these gene products from other zyxin family members and potentially mediates functions specific for WTIP.

WTIP Expression—WTIP tissue expression was analyzed on an RNA master blot (not shown). WTIP is expressed in a pattern similar to that reported for WT1 during embryonic development (2, 11, 22). WTIP transcripts are identified in kidney, lung, eye, and ovary. Consistent with overlapping tissue expression patterns between WT1 and WTIP, WT1 is required for normal retinal (23) as well as genitourinary (11) development. WT1 mutations in humans are associated with pulmonary dysplasia and mesangial sclerosis (24). In addition, WTIP is expressed in thyroid and salivary gland, tissues that, like kidney, undergo a mesenchymal to epithelial transition during ontogeny. By Northern analysis (Fig. 3A), WTIP is a 2.2- and 2.0-kb transcript in human and mouse kidney, respectively. Northern blots (Fig. 3A), reverse transcription-PCR (Fig. 3B) and in situ hybridization (not shown) also show that undifferentiated and differentiated murine podocytes contain WTIP mRNA. We next evaluated WTIP expression during kidney development. WTIP mRNA is robustly expressed during mouse nephrogenesis in a temporal pattern similar to WT1 (Fig. 3C). Peak WTIP mRNA expression is at E16–E17 and persists into adulthood (Fig. 3C), although at lower levels, consistent with restriction to podocytes. In situ hybridization localized WTIP transcripts to avascular mesenchymal condensates and podocytes in developing S-shaped bodies in E15 kidneys (Fig. 3D).

WTIP Shuttles between Nucleus and Cytosol—To understand how WTIP could regulate WT1 transcriptional activity, we determined the subcellular localization of WTIP by ectopically expressing tagged WTIP constructs in COS7 cells. Myc-tagged N/H9004 WTIP (amino acids 1-185), the sequence identified in the yeast two-hybrid assay that only contains the LIM domain-containing region of WTIP, co-localized with WT1 in nuclei (Fig. 4A), as expected for a protein that regulates activity of a transcription factor. In contrast, full-length WTIP localized in the cytosol and at sites of cell-cell contact (Fig. 4B), a surprising finding given that WTIP was discovered as a partner for a transcription factor. Zyxin and related molecules, including thyroid receptor-interacting protein 6, lipoma partner protein, and ajuba, shuttle from cytosolic structures to the nucleus (25–28), a process that requires Crm1-mediated nuclear export. The pre-LIM region of WTIP, deleted in N/H9004 WTIP, contains a consensus nuclear export sequence (13-AALLLAGLGLRES) (Fig. 2B), with a core of hydrophobic residues required for recognition by the Crm1 export receptor. We next determined WTIP expression in COS7 cells by immunofluorescence both before and after treatment with leptomycin B, an inhibitor of Crm1-mediated nuclear export (Fig. 4C). WTIP was present in the cytosol of COS7 cells at base line (Fig. 4C, left panel) and co-localized with FLAG-WT1 (not shown). By 6 h, WTIP nuclear localization was equivalent to that observed in COS7 cells containing N/H9004 WTIP (Fig. 4, compare C (right panel) with A). These data suggest WTIP normally localizes in the cytosol and functions as a communication signal between cytosolic structures and the nucleus.

WTIP Co-precipitates with WT1 in COS7 Cells and Represses WT1-dependent Transcription—Because false-positive interactions could arise from the yeast two-hybrid screen, we confirmed that WT1 and WTIP associate in COS7 cells using yeast two-hybrid assay. ClustalW alignment of mouse WTIP (GenBankTM XP_059037.3) and human WTIP (GenBankTM AAH54125.1) sequences (ClustalW, UCSD Supercomputer, available on the World Wide Web at workbench.sdsc.edu/).
immunoprecipitation and GST pull-down assays. Extracts from COS7 cells co-transfected with Myc-tagged N\!/H9004 WTIP and FLAG-WT1 were immunoprecipitated with anti-FLAG or anti-WT1 antibodies and probed by Western blotting with anti-Myc antibodies. myc-N\!/H9004 WTIP is co-precipitated by both anti-WT1 and anti-FLAG antibodies (Fig. 5A). Under normal circumstances, WTIP does not co-precipitate with WT1 (Fig. 5B), consistent with WTIP exclusion from the nucleus (Fig. 4B). However, WTIP and WT1 did co-precipitate with WTIP when nuclear export is blocked by leptomycin B (Fig. 5B). Neither full-length zyxin nor a zyxin construct (N\!/H9004 zyxin; amino acids 368–565) that localizes to the nucleus (not shown) (25) co-precipitated with WT1 (Fig. 5C). Pull-down assays, using a GST-N\!/H9004 WTIP fusion protein or GST-IL1 receptor cytosolic domain (IL1Rcd) fusion protein as a negative control, confirmed WT1 specifically associated with WTIP (Fig. 5D).

We next determined whether WTIP regulated WT1 transcriptional activity, using an amphiregulin promoter-luciferase reporter assay. Amphiregulin is an authentic WT1 transcriptional target in vivo, and its promoter contains a specific 10-bp binding element necessary for WT1-dependent transcriptional activation (Fig. 6A) (15). WTIP sequences, when localized in the nucleus, specifically repressed WT1-dependent transcription (Fig. 6B). Luciferase activity was stimulated between 3- and 10-fold in 3T3 (Fig. 6B, left panel) and HeLa cells (Fig. 6B, middle and right panels), respectively, transfected with a WT1 expression vector and the amphiregulin promoter-reporter construct (pGL2-B) versus cells transfected with WT1 and an amphiregulin promoter whose WT1 response element is mutated (pGL2-B\!/H9004 WRE) (Fig. 6B). When N\!/H9004 WTIP is co-expressed with WT1 and pGL2-B, WT1-dependent luciferase activity diminished by 50–90% compared with cells transfected with WT1.
alone (Fig. 6, A and B). N\textsuperscript{A}WTIP alone did not activate transcription from pGL2-B. In contrast, neither N\textsuperscript{A}zyxin (Fig. 6B, right panel) nor full-length zyxin (not shown) regulated WT1-dependent transcriptional activity. These studies demonstrate that WTIP and WT1 specifically associate in nuclei and that WTIP represses WT1-dependent transcription via its LIM domains.

**WTIP Links Podocyte Adherens Junction Proteins with Sites of Actin Assembly**—We next immunolocalized Myc epitope-tagged, full-length WTIP, ectopically expressed in cultured podocytes. Myc-WTIP appeared to localize in differentiated podocytes at arborized projections, at cell edges, and in cytoplasmic spots (Fig. 7A). Since WTIP spots were reminiscent of actin spots, which have been characterized as sites of dynamic F-actin assembly (29), we next assessed if WTIP co-localized with actin structures. WTIP was not found at the end of actin stress fibers (not shown) but did co-localize with stress fiber-independent actin spots in cytochalasin D-treated 3T3 cells (Fig. 7B) and podocytes (not shown).

CD2AP was originally identified as an adapter protein that links the cytoplasmic domain of CD2, a T cell adhesion protein, with the actin cytoskeleton and associates with stress fiber-independent F-actin spots after cytochalasin treatment (30). We hypothesized that CD2AP could link WTIP and the actin...
WTIP did not co-localize with Mena in these adhesion complexes, consistent with lack of localization of WTIP and β1 integrin and vinculin (not shown). These data suggest that WTIP appears to be part of a multiprotein complex that connects actin assembly complexes to the slit diaphragm proteins.

**DISCUSSION**

Podocytes arise from the metanephric mesenchyme after a tightly orchestrated differentiation program to produce a highly specialized phenotype. In vitro, animal and human data suggest that podocyte dysregulation can initiate and/or perpetuate progressive glomerular scarring (35). In proteinuric diseases, regardless of the etiology, podocytes undergo marked morphologic changes (36). The actin cytoskeleton rearranges into a cytoskeletal mat below the plasma membrane opposed to the GBM, slit diaphragm structures are lost, and the podocyte assumes a cuboidal shape (36). At a molecular level in both human biopsies and experimental models, this stereotypical morphological response is associated with changes in cytoplasmic, plasma membrane, and nuclear podocyte differentiation marker expression. Given its unique microenvironment with exposure to hemodynamic forces and high flow of ultrafiltrate, the podocyte must be able to respond rapidly to changes in physical forces or soluble signals, processes that undoubtedly involve dynamic regulation of actin cytoskeletal organization in the short term and changes in gene expression in the longer term. An increasing number of molecules are known to shuttle between cytoplasm and nucleus to regulate gene expression. We speculated that specific molecules might transmit information from the filtration barrier to the nucleus to allow the podocyte to regulate its differentiation state in response to environmental signals.

Molecular mechanisms, which regulate podocyte cell fate decisions, involve changes in gene expression. Data derived from genetically modified mice and families with syndromes including glomerular disease have identified several transcription factors required for podocyte specification and differentiation, including WT1 (1, 5). To identify proteins that regulate podocyte phenotype, a kidney library was screened in a two-hybrid assay with WT1 as bait. We identified a novel protein, WTIP, that has three LIM protein-protein interaction domains and is similar to the zyxin family of adhesion complex molecules. The tissue expression pattern of WTIP and WT1 overlapped. WTIP contains a nuclear export signal, and export is inhibited by leptomycin B. If WTIP is retained in the nucleus, it physically associates with WT1 and inhibits WT1-dependent transcriptional activation of the amphiregulin promoter. Thus, WTIP appears to be a previously undiscovered regulator of podocyte phenotype, which modulates WT1-dependent gene transcription.

Pathways that regulate WT1 function remain incompletely defined. WT1 interacts with a number of proteins (11). WT1 and the transcriptional co-activator CREB-binding protein directly interact through the first two zinc fingers of WT1 and the E1A-binding domain of CBP, leading to the synergistic interaction of the amphiregulin promoter (37). Par-4 (prostate apoptosis response factor) can co-activate or co-repress WT1-dependent transcription by interacting with either the 17-amino acid exon 5 region or the zinc finger region, respectively (38, 39). Ciao1, a novel WD domain-containing protein, and p53 family of proteins also inhibit transcription by WT1 (40, 41). Another LIM domain-containing protein, FHL2, physically interacts with WT1 to modulate transcription of genes important in gonadal differentiation (42). WTAP (WT1-associating protein) also was identified through yeast two-hybrid screening and partially co-localizes in nuclear spiculesomes, but its function is unknown (43). Although persistent expression of WT1 in
podocyte nuclei suggests that podocyte differentiation requires ongoing transcription of WT1-dependent genes, none of the known WT1 protein partners explains how WT1 activity is regulated in the differentiated podocyte. WTIP may represent such a protein.

The domain structure of WTIP suggests it is a scaffold molecule. Our data demonstrate that, in the absence of leptomycin, WTIP co-localized with CD2AP in stress fiber-independent actin spots, sites of dynamic actin assembly in other cells, and with Mena at sites of developing cell-cell contacts. These data suggest that WTIP is necessary for normal podocyte differentiation by organizing proteins that form cell-cell contacts between foot processes and that WTIP is excluded from nuclei except to communicate signals between the two cellular compartments. Three carboxyl-terminal LIM domains and SH3 and PDZ binding sites represent the protein-protein interaction modules within WTIP, a domain structure closely related to the zyxin family of LIM domain-containing proteins.

Fig. 5. A, COS7 cells were transfected with pCMVTag-mycNWTIP, pCMVTag-FLAGWT1 (−KTS), or pCMVTag (vector control) as indicated. Protein expression was verified by Western analysis of cell extracts (250 mM NaCl, 0.5% Triton, and 0.1% deoxycholate) (left panel). Lysates were subjected to immunoprecipitation (IP) with anti-FLAG agarose or anti-WT1 antibody (C19; Santa Cruz Biotechnology), and blots were probed for myc-WTIP using anti-Myc antibody (A14; Santa Cruz Biotechnology) (right panel). IB, immunoblot. B, COS7 cells were cotransfected with pCMVTag-mycWTIP and pCMVTag-FLAGWT1 (−KTS) and incubated in the absence or presence of leptomycin B (6 h, 10 ng/ml). Protein expression was verified by Western analysis (left panel). Cells lysates were subjected to immunoprecipitation with anti-FLAG-agarose and probed for myc-WTIP. WTIP, when retained in the nucleus after leptomycin treatment, co-precipitates with WT1 (lane 4) but is not detectable when the Crm1 nuclear exporter remains functional (lanes 1 and 2). C, the physical association of WTIP and WT1 is specific. COS7 cells were transfected with pCMVTag-mycNWTIP, pCMVTag-mycNWTIP, pCMVTag-mycNWTIP, pCMVTag-FLAGWT1 (−KTS), or pCMVTag as indicated. Expression of transfected proteins was verified by Western analysis using anti-Myc antibody (A14) and anti-WT1 antibody (C19) (left panel). Lysates were subjected to immunoprecipitation with anti-FLAG-agarose, and blots were probed for WT1-associated Myc-tagged proteins. WT1 only co-precipitated with NWTIP. D, GST pull-downs confirm that WTIP specifically associated with WT1. COS7 cells were transfected with pCMVTag-FLAGWT1 (−KTS) or empty vector (control). Western blotting (left panel) verified WT1 expression in transfected cells (left panel). Cell lysates were incubated with GST and GST-ILRCD or GST-NWTIP (5 μg each), and associated proteins were captured with glutathione-Sepharose. WT1 was detected by Western blotting with anti-WT1 antibody (C19; Santa Cruz Biotechnology). Only GST-NWTIP precipitated FLAG-WT1 from lysates.
family contains both human and murine WTIP and the *Drosophila* orthologue (21) and is characterized by proline-rich amino termini and three LIM protein-protein interaction domains. The LIM domain is a conserved zinc finger protein interaction motif, and proteins containing LIM domains mediate cytoskeletal organization, cell lineage specification, and organogenesis and oncogenesis (44, 45). Zyxin and related LIM domain-containing proteins shuttle from sites of cell adhesive interactions to the nucleus and can regulate cell differentiation state (27, 28, 46). In addition to zyxin-like molecules, other junctional proteins also translocate to the nucleus and bind to transcription factors, including ZO-1, Cask/Lin-2, β-catenin, and human ASH1 (47–49). These data suggest that cell junctions, like the podocyte slit diaphragm, function as switching stations, containing molecules that can transmit information to the nucleus and regulate cell responses to extracellular stresses.

Recent genetic and biochemical studies have begun to define

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**A.**

*Amphiregulin* Promoter Construct:

|       | WRE       | CRE       | TATA     | LUCIFERASE |
|-------|-----------|-----------|----------|------------|
| -328  |           |           |          | pGL2-B:    |
|       |           |           |          | WRE: 5-CCGTGGGTGG-3 |
| -87   |           |           |          | pGL2-ΔA:   |
|       |           |           |          | WRE: 5-XXXGGGTGG-3 |

**B.**

![Graphs showing luciferase activity](image-url)
the molecular architecture of the filtration barrier, which requires elaboration of podocyte foot processes and assembly of cell-matrix and specialized cell-cell junctions, the slit diaphragm, for normal function. The slit diaphragm is a modified adherens junction that contains proteins that have been discovered by positional cloning (\textit{NPHS1} (nephrin), \textit{NPHS2} (podocin), and \textit{FSGS1} (\textit{H9251}-actinin-4)) and by serendipity (CD2-associated protein CD2AP). Nephrin, an immunoglobulin superfamily member, is thought to be a major transmembrane component of the slit diaphragm (50). Podocin, which is similar to stomatin family scaffold molecules, has a single hairpin-like structure with both the N-terminal and C-terminal domains in the cytosol (51). CD2AP localizes in stress fiber-independent actin spots after treatment with cytochalasin D (middle panel). The merged image shows co-localization of CD2AP and WTIP in spots (right panel). Bar, 10 μm.

FIG. 7. A, differentiated podocyte transfected with myc-WTIP and stained with the anti-Myc antibody (9E10). WTIP expression is observed in arborized projections and at the plasma membrane periphery (arrows). Perinuclear staining represents protein overexpression in the endoplasmic reticulum. Nuclear staining is absent. B, NIH3T3 cells were transfected with myc-WTIP, treated with cytochalasin D (1.5 μg/ml, 30 min), and stained for actin fibers (red, phalloidin, left panel) and Myc (green, middle panel). WTIP co-localizes with stress fiber-independent actin spots. C, differentiated podocytes were transfected with full-length myc-WTIP, treated with cytochalasin D, and stained for endogenous CD2AP (green, middle panel) and Myc (red, left panel). CD2AP localizes in stress fiber-independent actin spots after treatment with cytochalasin D (middle panel). The merged image shows co-localization of CD2AP and WTIP in spots (right panel). Bar, 10 μm.

FIG. 8. WTIP co-localizes with Mena. Differentiated podocytes were transfected with myc-WTIP and stained for endogenous Mena (green, A) and for Myc (red, B). C shows the merged image. Nuclei are identified with 4',6 diamidino-2-phenylindole (DAPI) (blue). WTIP co-localizes with Mena at cell borders (yellow), suggesting that WTIP may play a role in organizing cell-cell junctions. Size bar, 10 μm.

FIG. 9. A model for WTIP function in the podocyte. In normal glomeruli, WTIP is part of a multiprotein complex in the foot process and may link the CD2AP/nephrin/podocin complex to adherens junction proteins by regulating actin assembly. After injury, WTIP translocates into the nucleus, where it represses WT1-dependent gene expression to permit simplification of podocyte phenotype. Loss of WTIP from its cytosolic location also promotes redistribution of slit diaphragm proteins and actin rearrangement characteristic of foot process effacement.

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with actin cytoskeleton. ZO-1 and α-catenin also interact with F-actin (34), suggesting that submembranous actin cytoskeleton is essential for maintaining the podocyte foot process structure and adhesion complex assembly. Foot process retraction is the stereotypical podocyte response to injury and is characterized both in vivo and in vitro by marked actin cytoskeletal rearrangement (58), observations further supporting a significant role for actin as a central organizer of podocyte foot process architecture. ACTN4, which encodes the actin cross-linking protein α-actinin-4, was identified as a candidate gene locus on chromosome 19q13 mapped from three families with focal glomerulosclerosis. Like CD2AP, α-actinin-4 associates with the actin cytoskeleton and must be important in maintaining the filtration barrier, since mutations in ACTN4 cause focal glomerulosclerosis (59). Human ACTN4 mutations generate a phenotype similar to mutations in NPHS1, NPHS2, α-intergrin, NEPH-1, CD2AP, and podocalyxin that have been reported in humans or mice, suggesting that actin organization is a critical link to proper assembly of podocyte slit diaphragm proteins (31). Disruption of the actin cytoskeleton with cytochalasin D abolishes adherens junction assembly (33, 34).

Our data demonstrate that WTIP is expressed in podocytes in culture and in vivo. Ectopically expressed, epitope-tagged WTIP co-localizes with CD2AP and Mena, a protein associated with cadherin-based cell contacts, and in proximity to podocyte actin spots. WTIP contains a nuclear export signal, and its nuclear export is inhibited by leptomycin B. If WTIP is retained in the nucleus, WTIP physically associates with WT1 and inhibits WT1-dependent transcriptional activation of the amphi- gene locus on chromosome 19q13 mapped from three families with focal glomerulosclerosis. Like CD2AP, WTIP co-localizes with CD2AP and Mena, a protein associated with cadherin-based cell contacts, and in proximity to podocyte actin spots. WTIP contains a nuclear export signal, and its nuclear export is inhibited by leptomycin B.

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