Supplemental Results.

Characterization of human podocytes expressing WTIP-V5 under control of a TCN-inducible promoter (GEC-WTIP-V5). Supplemental Fig S1A demonstrated that differentiated GEC-WTIP-V5 express podocyte markers, the actin filament binding protein synaptopodin and the transcription factor WT1. Addition of TCN overnight robustly induced WTIP-V5 expression in differentiated podocytes, which persisted for at least 48 hr after replacement of TCN-containing medium with fresh medium (Fig S1B) and reflecting the $T_{1/2}$ of TCN. Cell viability was determined by MTT assay (see Supplemental Methods) after 24 hrs exposure to increasing concentrations of LPS (0.05 μg/ml, 0.1 μg/ml, 0.25 μg/ml, 0.5 μg/ml, 1.0 μg/ml and 2.5 μg/ml) and compared with time-matched control (Fig S1C). The MTT assay shows that addition of LPS to GEC-WTIP-V5 has no effects on cell viability. Taken together, these data demonstrate that GEC-WTIP-V5 express podocyte markers after differentiation and inducibly express WTIP-V5 in podocyte cell-cell contacts, as demonstrated by colocalization with the adherens junction proteins, β-catenin and ZO-1 (Fig S1D).

Characterization of anti-WTIP peptide antibody specificity. To initially test specificity of the affinity-purified anti-WTIP antibody (details in supplemental "Materials and Methods"), we immunoblotted purified lysates from E. coli expressing either glutathione S-transferase (GST)-tagged NΔWTIP (Coomassie stain shown in Fig S5A, lane 1) or lysates from cultured murine podocytes. The WTIP antibody detected a single band of the appropriate size in the lysates from bacteria expressing GST-NΔWTIP (Fig S5A, lane 2) and podocytes (lane 3). The podocyte signal could be competed by pre-incubating the anti-WTIP peptide antibody with excess GST-NΔWTIP fusion protein, which contains the WTIP peptide immunogen sequence (Fig S5A, lanes 3-5). Myc epitope-tagged LIMD1, zyxin or ajuba, members of a subfamily of LIM domain proteins that also translocate from sites of cell adhesion into the cell nucleus (1,2), were immunopreciptiated from COS7 cells transfected with the indicated expression constructs. The immunoprecipitates were analyzed by immunoblotting with an anti-myc antibody and the resulting blot demonstrated bands of predicted size for each fusion protein (Fig S5B, left panel). In contrast, no signal was detected when immunopreciptates from the same cells were probed with anti-WTIP antibody (Fig S5B, right panel); the anti-WTIP antibody did detect a band of appropriate size in immunopreciptates from COS7 cells expressing a GFP-WTIP fusion protein (Fig S5B, right panel). We further explored specificity of the anti-WTIP antibody for endogenous mouse podocyte WTIP (Fig S5C). In this experiment, endogenous LIMD1 was immunoprecipitated from podocyte lysates with anti-LIMD1 antibody (gift of Dr. Greg Longmore, Washington University School of Medicine, St. Louis, MO (3)). LIMD1 was identified in Western blots of podocyte lysates and anti-LIMD1 immunoprecipitates when probed with the anti-LIMD1 antibody (Fig S5C, experiment (EXP) 1, left panel). The same blot was stripped, probed with the anti-WTIP peptide antibody and overexposed to visualize remnant signal from the anti-LIMD1 immunoblot (Fig S5C, EXP 1, right panel). As expected, reblotting with the anti-WTIP peptide antibody identifies a band clearly distinguishable from LIMD1 in podocyte whole cell lysates but not the lane loaded with anti-LIMD1 immunoprecipitates, further confirming specificity of the anti-WTIP peptide antibody. In a second experiment, the anti-WTIP peptide antibody only detected a protein in podocyte lysates but not in a lane loaded with anti-LIMD1 immunoprecipitates (Fig S5C, EXP 2). As a final test of the specificity for the anti-WTIP peptide antibody, we probed lysates of podocytes stably expressing control shRNA or two different WTIP shRNA lentiviral vectors. Podocyte WTIP was detected by the anti-WTIP peptide antibody in podocytes expressing controlled shRNAs but not in podocytes expressing the WTIP shRNAs (Fig S5D). Probing the same blots with anti-β-actin demonstrated equivalent protein loading. Taken together, these data show the anti-WTIP peptide antibody used in this study is specific for WTIP and does not recognize epitopes on closely related members of the Ajuba sub-family of LIM domain containing proteins.

Supplemental Materials and Methods.

Materials. Lipopolysaccharide (Escherichia coli 0111:B4), cytochalasin D, and aurintricarboxylic acid (AA) were purchased from Sigma (St. Louis, MO). JNK inhibitor II (SP600125), EHNA hydrochloride and p38 inhibitor (SB202190) were purchased from Biosource (Camarillo, CA). Nocodazole was purchased from Tocris Bioscience (Ellisville, MO). The primary antibodies used were: rabbit polyclonal anti-GFP and mouse monoclonal anti-GFP (JL8, Clontech, Mountain View, CA), anti-lamin B2 and anti-ZO-1 (Zymed, South San Francisco, CA), anti-β-actin and anti-FITC-V5 (Invitrogen, Carlsbad, CA), anti-tetracycline (MoBiTec, Göttingen, Germany), rabbit polyclonal anti-p-JNK, anti-JNK, anti-p-p38, and anti-p38 (Cell Signaling, Danvers, MA), anti-c-myc 9E10, rabbit polyclonal anti-rho-GDI, and anti-histone H3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-dynein intermediate chain (DIC; Chemicon International, Temecula, CA), and anti-β-catenin (BD Transduction Laboratories, San Diego, CA). The rhodamine-phalloidin was purchased from Molecular Probes (Carlsbad, CA).

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. MTT assay was employed to quantify the number of viable cells. Briefly, 100 µl of the cell suspension or 1×10^4 cells were seeded into 6-well tissue-culture plates, allowed to adhere for 24 hrs and then treated with various concentrations of LPS (0.05 µg/ml, 0.1 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1.0 µg/ml and 2.5 µg/mL). Control cells were not treated with LPS. After treatment with LPS for the indicated periods, 10 µl MTT was added into each well for a final concentration of 0.5 g/L, and incubated for 4 hrs. The formazan crystals produced by viable cells were dissolved by Me_2SO. The optical density (OD) of the solution was measured at 490 nm. Cell viability is directly proportional to OD value. The viable cell number was expressed as a percentage of control cells and measured as $OD_{LPS\text{-}treated}/OD_{control}\times100\%$.

Transient transfections. The indicated expression vectors for WTIP, WTIP with deletion of the pre-LIM domain (NΔWTIP, amino acids 186-430), LIMD1 and zyxin were generated by cloning the cDNA for the protein coding regions into pCMV-Tag (Stratagene, LaJolla, CA) in frame with a N-terminal GFP- or myc tag as we previously described (4). COS7 cells were transiently transfected with the indicated expression vector using Superfect reagent (Qiagen, Valencia, CA) according to manufacturer's protocol. Briefly, cells were incubated 2.5 hrs with Superfect:DNA complex at a ratio to 1:5 in serum-free DMEM after which media was changed to DMEM containing 10% FBS. Cells were harvested 48 hrs after transfection and analyzed by immunoblotting or immunoprecipitation with the indicated antibodies.

Immunoprecipitation and immunoblotting. GEC-WTIP-V5 were transiently transfected using Fugene6 reagent (Roche, Mannheim, Germany) with pcDNA3.0 Flag-JIP3 construct, a generous gift from Dr. Roger Davis, and grown to 80% confluence on 10 cm tissue culture plates. Cells were rinsed with PBS containing protease cocktail inhibitor tablet, scraped, and pelleted. The cell pellet was resuspended in 800 µl of immunoprecipitation (IP) buffer (50 mM TrisHCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 2 mM Na_3VO_4, 2 mM NaF, 1% TritonX-100, 10% glycerol). Following centrifugation to remove debris, supernatants were matched for protein, precleared with protein A-Sepharose and incubated for 3 hrs at 4^o C with either non-immune mouse IgG, mouse-anti-V5 antibody (1:1000 dilution), anti-FLAG antibody M2 (1:2000), or mouse anti-DIC antibody (1:1000) and incubated overnight at 4^o C with 25 µl of a 50:50 slurry of protein A Sepharose. The antibody-antigen complexes were captured by centrifugation (960xg, 10 min, 4^oC) and washed with IP buffer (3X). Bound proteins were released by boiling in Laemmli buffer for 5 min. Eluted proteins were separated by 4-20% SDS polyacrylamide gels (Invitrogen), transferred to nitrocellulose membranes (Millipore, Billerica, MA), and analyzed by Western blotting using either a monoclonal anti-V5 antibody (1:5000), anti-FLAG antibody M2 (1:7500), or monoclonal anti-DIC antibody (1:1000) and a mouse horseradish peroxidase (Amersham). Immunoreactive proteins were identified by enhanced chemiluminescence and autoradiography (Amersham). To quantify the bands obtained via Western blot analysis, we applied ImageJ software based analysis (http://rsb.info.nih.gov/ij/). The area under curve (AUC) of the specific signal was corrected for

the AUC of the loading control. The value for the control ('Solv') condition was set as 1 and other conditions were recalculated correspondingly to allow ratio comparisons.

RNA isolation and RT-PCR. Total RNA was isolated from normal cultured mouse podocytes and mouse kidney as previously described (4). Briefly, 2-4 μg of total RNA were reverse transcribed in the presence of random hexamers and RT according to the following sequence, 65^{o}C for 5 min followed by 50^{o}C for 60 min and 75^{o}C for 15 min. Sample cDNA (1 μl) was used to perform each 25 μl PCR reaction. Conditions for PCR were are follows: 95^{o}C for 4 min; 95^{o}C for 30 sec, 53^{o}C for 30 sec, 72^{o}C for 1 min (30 cycles); 72^{o}C for 10 min, 4^{o}C hold. PCR products were visualized in 1% TBE agarose gel. Primer pairs for JSAP1 (JIP3): Forward 5'- GTG ACA AGG CCG CCA GTA GTT TC – 3' and Reverse 5' – GAC AAT CTT GGG TTG ACG GGT ATT T – 3'.

Preparation of affinity column and affinity purification of anti-WTIP antibody. An antibody was raised in rabbits immunized with a WTIP peptide that is identical in mouse and human WTIP (SAAERRLEALTRELERALEARTAR; human WTIP [NP_001073905], amino acids: 196-219; mouse WTIP [NP_997095], amino acids: 164-187). The anti-WTIP antibody was affinity purified from crude serum as follows. The WTIP peptide immunogen was dissolved in the coupling buffer (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl). CNBr-activated Sepharose 4B medium was prepared by resuspending the freeze dried powder in 1 mM HCL. The medium was washed for 15 min with 1 mM HCl on a sintered glass filter. The coupling solution was mixed with the immunogen and medium in a stopped vessel and rotated end-over-end overnight at 4°C. The excess ligand was washed with at least 5 gel volumes of coupling buffer. Remaining active groups were blocked by transferring the medium to 0.1 M Tris-HCl buffer, pH 8.0, and let stand for 2 hrs. The coupled medium was washed 3x sequentially with 0.1 M acetate buffer, pH 4.0 and 0.1 M Tris-HCl, pH 8.0. The coupled column was incubated with rabbit antiserum overnight at 4°C and washed sequentially at 1 ml/min with 20 ml of each of the following solutions: (i) TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl); (ii) 0.1 x TBS. After the last wash, anti-WTIP antibody was eluted. The bound proteins were eluted with 2 ml of 0.1 M glycine-HCl, pH 2.5. Fractions were collected in tubes preloaded with 250 μl of 1 M Tris base. After each fraction volume reached 1 ml, it was mixed to neutralize the eluate. The column was regenerated immediately after use by washing the column with 40 ml of 1 M Tris-HCl, pH 8.0 and stored in TBS containing 0.02% sodium azide at 4°C.

LPS-induced FP effacement and albuminuria. All animal studies were approved by Case Western Reserve University IACUC. C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Base-line urine albumin excretion of 4- to 6-week-old female C57BL/6 mice (n = 25 mice in total, n = 11, PBS injected; n = 14, LPS injected), was determined by urine dipstick (Albustix; Bayer, Tarrytown, New York, USA) prior to intraperitoneal injection of either 200 μg LPS (1 mg/ml in sterile LPS-free PBS) in a total volume of 200 μl, or an equal volume of sterile, LPS-free PBS as vehicle control. Urine dipstick albuminuria (Albustix) was measured and confirmed transient proteinuria in the mice, which resolved by 72 hrs following LPS injection (n = 25 mice in total, n = 11, PBS injected; n = 14, LPS injected; base line, 6 ± 5.45 mg/dL vs. LPS 6 hrs, 8 ± 4.47 mg/dL, LPS 12 hrs, 44 ± 31.30 mg/dL, LPS 24 hrs, 2200 ± 447.21 mg/dL , LPS 48 hrs, 220 ± 109.55 mg/dL, LPS 72 hrs, 36 ± 37.14 mg/dL; *, $P<0.05$). Urine osmolality was unchanged after LPS injection, indicating that the increasing albuminuria was not a reflection of urinary concentration. Kidneys were harvested and processed for immunofluorescence. All measurements were performed in triplicate.

*Co-localization analysis***.** Using unbiased strategies in ImageJ, confocal microscopic images of mice treated with LPS or PBS were analyzed for co-localization of WTIP and the nuclear marker TOPRO-3 (W. S. Rasband, National Institutes of Health, Bethesda, Md., 1997-2004, http://rsb.info.nih.gov/ij/). Images were acquired in channels optimized to detect FTIC and TOPRO-3 fluorescence, corresponding to anti-WTIP staining and nuclear staining, respectively. The light paths for the two filter sets were adjusted

to ensure the alignment of light emitted from a point in the glomerulus through different filter sets. Background was corrected using the background subtraction function on ImageJ. The glomerulus was selected as the region of interest in both green and blue channels. Areas that contained staining artifacts, as indicated by intense regions of fluorescence in both channels, were excluded from analysis. Co-localization analysis on a glomerular image plane was assessed using two methods. First, the Co-localization Threshold plug-in, which is included in the WCIF version of ImageJ (http://www.uhnresearch.ca/facilities/wcif/imagej/) (5) and described in detail in (6), was used. This function uses the following principle. When two proteins are spatially distributed over a region of interest, no specific co-localization exists between the two proteins when their spatial distributions are independent of each other, although some amount of random overlap will be present between the two images. Conversely, if the two proteins have a specific co-localization, then the overlay of the two spatial distributions will show a level of correlation that is in excess of the random overlap. A scatterplot was generated with the green channel on the x-axis and the blue channel on the y axis. A threshold for co-localization is calculated from an orthogonal regression line and is defined as the point when Pearson's correlation coefficient for green and blue pixels equals zero. A grayscale image that includes only co-localized pixels is then generated. These co-localized pixels were pseudocolored green. A second method, Intensity Correlation Analysis (ICA) (7), was used to confirm the results of the Colocalization Threshold. ICA calculates the Product of the Differences from the Mean (PDM) value for each pixel: PDM = (blue intensity- mean blue intensity) × (green intensity – mean green intensity). The "Display +ves Option Only" in ImageJ generates an image, which displays positive PDM values resulting from area within the glomerulus in which both green and blue pixels exceed the mean (i.e. blue intensity-mean blue intensity and green intensity-mean green intensity are both positive), which were psuedocolored white.

shRNA knockdown of mouse WTIP. Control and mouse WTIP shRNAs cloned into the HIV-based lentiviral vector, pLKO.1 were purchased from Open Biosystems (Huntsville, AL): pLKO.1-TRC, an empty vector control that contains a 18 nt stuffer sequence (RHS4080); pLKO.3G-TRC eGFP, which contains an eGFP marker and is unrelated to the target WTIP sequence (RHS4459); pLKO.1-TRC WTIP1, which targets mouse Wtip [NM_207212] nt 1471-1491 (TRCN0000095769): 5'-CCGGCCCGCAACAAAGAAGCGATTTCTCGAGAAATCGCTTCTTTGTTGCGGGTTTTTG-3'; or pLKO.1-TRC WTIP2 targets mouse WTIP [NM_207212] nt 766-786 (TRCN0000095770): 5'-CCGGCGCGAGACTACTTTGGCATTTCTCGAGAAATGCCAAAGTAGTCTCGCGTTTTTG-3'. Details for each vector are available at http://www.openbiosystems.com/Query/?i=0&q=WTIP. Protocols for culturing, plasmid prep, virus production and transduction of lentiviral shRNA constructs can be accessed from the following link: http://www.broad.mit.edu/genome_bio/trc/publicProtocols.html. Briefly, 1 µg of retroviral DNA, 1 µg packaging plasmid (pHR'CMV8.2deltaR), 1 µg packaging (pHR'8.2deltaR) at a 8:1 ratio with envelope plasmid (pCMV-VSV-G), 94 µl total of DME without serum, and 6 µl of FUGENE were mixed and incubated for 20 min at RT. Complexes were added to $5x10^5$ 293T cells in 6 cm^2 dishes containing 5 mL of media. Following 3 days of incubation, medium from the 293T cells was collected and filtered using a 0.45 µm syringe filter. Infection of mouse podocytes was carried out for 16 hrs and virus was removed and replaced with fresh media. On day 7, 1 µg/ml of puromycin was added to the podocytes for stable selection and media was changed every 3 days for 15 days. WTIP protein knockdown was assessed by immunoblot analysis for endogenous WTIP expression using anti-WTIP antibody in whole cell lysates of mouse podocytes stably expressing the pLKO.1-TRC WTIP1 (sh-WTIP1) or pLKO.1-TRC WTIP2 (sh-WTIP2) compared to negative control pLKO.1-TRC (sh-EMP) and pLKO.3G-TRC eGFP (sh-GFP).

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Supplemental Figure Legends.

Fig S1: **Characterization of GEC-WTIP-V5 stable cell line**. **(A)** Characterization of differentiation at 37°C of GEC-WTIP-V5 cells by immunostaining with podocyte differentiation markers, the F-actin binding protein synaptopodin and the transcription factor WT1. Scale bars, 10 μm. **(B)** Immunoblot analysis of whole cell lysates of GEC-WTIP-V5 cells before and after the addition of tetracycline (1 μg/ml) overnight. TCN-containing medium was then removed and persistence of WTIP-V5 for the duration of our studies was documented (lanes labeled "wash-out). The same blot was stripped and reprobed with anti-tubulin antibody to assess protein loading. **(C)** Assessment of cell viability in GEC-WTIP-V5 podocytes incubated for 24 hrs with the indicated LPS concentrations. Viability was quantified using the MTT assay and normalized to untreated GEC-WTIP-V5 cells. Results are displayed as per cent of control, as described in the Supplemental "Materials and Methods". **(D)** Characterization of WTIP-V5 localization in GEC-WTIP-V5 cells by immunostaining with adherens junction markers β-catenin and ZO-1. Scale bars, 10 μm.

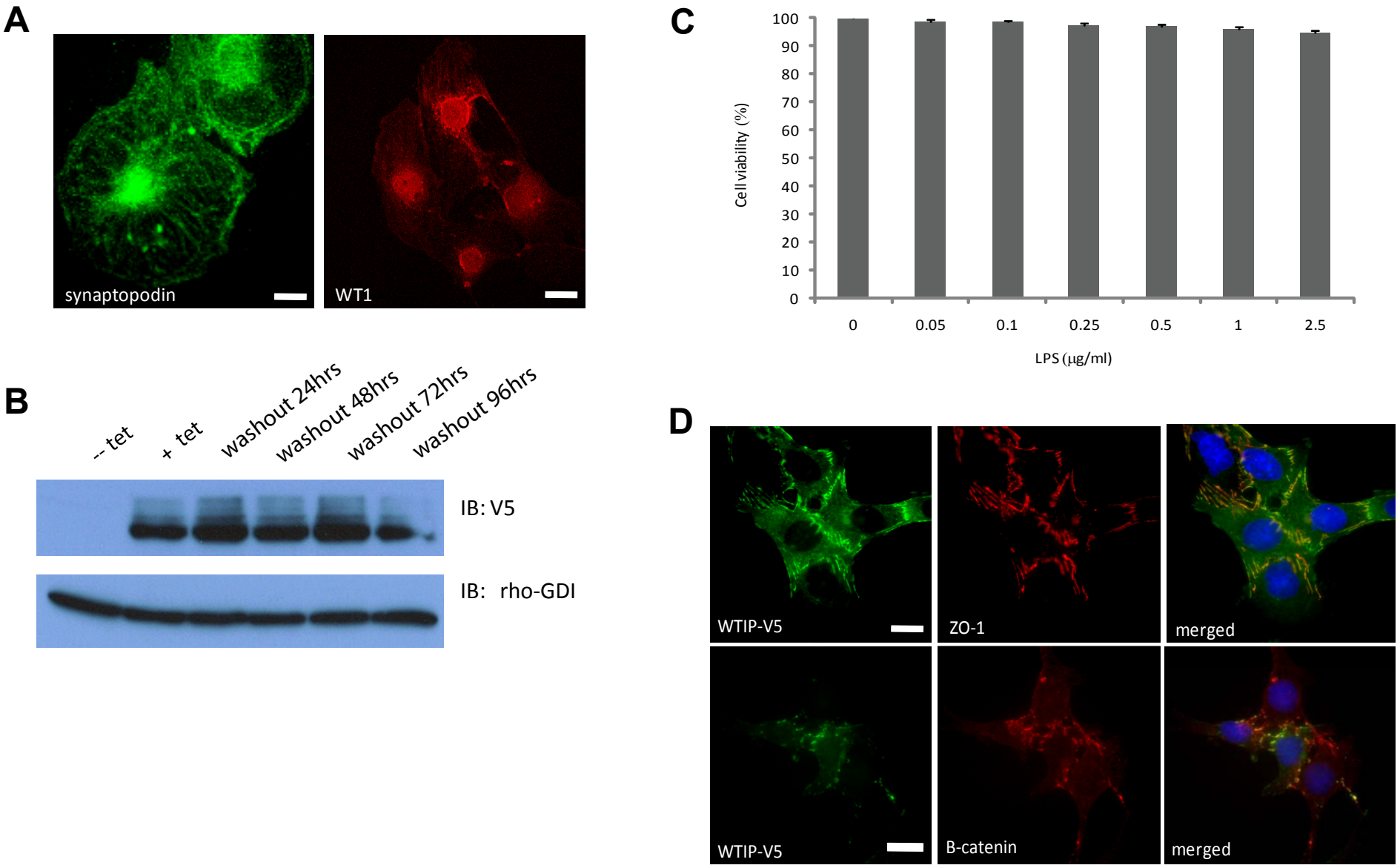
Fig S2. **Time course of WTIP-V5 localization following LPS treatment.** GEC-WTIP-V5 cells incubated with LPS (1 μg /ml) for 0-24 hrs at 37°C were fixed and stained with FITC-V5. Confocal images at the indicated time points demonstrate changes in WTIP-V5 localization over time following LPS treatment. Nuclei of some cells are outlined (white dashed lines). Scale bar, 10 μm.

Fig S3. **Screen of GFP-WTIP localization in various injury models**. **(A)** Confocal images of GFP-WTIP localization in mouse podocytes stably expressing GFP-WTIP treated with the indicated stimuli. Untreated control, LPS (1 μg/ml, 6 hrs), UV-C (50 mJ/m^2), H_2O_2 (50 μM, 6 hrs), or PAN (100 μg/ml, 24 hrs) treated were fixed and mounted with the nuclear marker DAPI. Scale bars, 10 μm. **(B)** Immunoblot analysis for JNK activation following various conditions of podocyte injury.

Fig S4. **Specificity of JNK and p38 inhibition. (A)** Immunoblot analysis of the effect of SP600125 and SB202190 on JNK and p38 phosphorylation, respectively. **(B)** Densitometric analysis was performed by normalizing phosphorylated p38 or JNK to total levels of p38 or JNK, respectively.

Fig S5. **Characterization of affinity purified anti-WTIP antibody. (A)** Lane 1 shows Coomassie stain of purified bacterial lysates expressing GST-NΔWTIP; lane 2 shows immunoblot of same lysate probed with anti-WTIP peptide antibody. The anti-WTIP peptide antibody recognizes a single band of appropriate size in podocyte lysates (lane 3), which is competed away by preincubating the antibody prior to blotting with excess GST-NΔWTIP fusion protein, as indicated (lanes 4 and 5). **(B)** COS7 cells were transiently transfected with expression vectors for the indicated N-terminal, myc-tagged LIM domain family proteins. Anti-myc immunoprecipitates were analyzed by blotting. Blots probed with anti-myc antibody identified fusion proteins of predicted sizes (left panel) but the anti-WTIP peptide antibody did not (right panel). In contrast, the anti-WTIP antibody identified an appropriately size protein in anti-GFP immunoprecipitates from COS7 cells transiently transfected with a GFP-WTIP expression vector (right panel). **(C)** Podocyte lysates (WCL) and anti-LIMD1 immunoprecipitates from podocytes were probed by immunoblotting with the indicated antibodies. Experiment (EXP) 1: Anti-LIMD1 recognized a protein of expected size in both WCL and anti-LIMD1 immunoprecipitates (left panel). This same blot was stripped, re-probed with anti-WTIP antibody and overexposed to detected residual signal from the anti-LIMD1 antibody. The re-probed blot clearly shows that the anti-WTIP antibody detects a protein distinct from LIMD1 (right panel). Experiment 2: Anti-WTIP detects a band of the appropriate size in podocyte WCL, but no protein is identified in anti-LIMD1 immunoprecipitates of podocyte lysates. **(D)** Mouse podocyte cells lines with stable knockdown of WTIP. Immunoblot analysis of whole cell lysates from podocyte cell lines stably expressing control lentiviral vectors, sh-EMP and sh-GFP, or lentiviral vectors containing mouse WTIP shRNAs, sh-WTIP1 and sh-WTIP2. The blot was re-probed with an anti-β-actin antibody to confirm equal protein loading (representative of 3 separate experiments). Densitometry was used to quantitate the efficacy of WTIP protein knockdown and results for WTIP signal normalized for β-actin expression (relative intensity) are shown in the bar graph. *$P < 0.05$, WTIP expression in podocytes expressing the WTIP shRNAs compared to podocytes expressing empty vector or irrelevant control shRNA vector with GFP

Figure S1.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C
Cell viability (%)
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70
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merged
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merged

Figure S2.

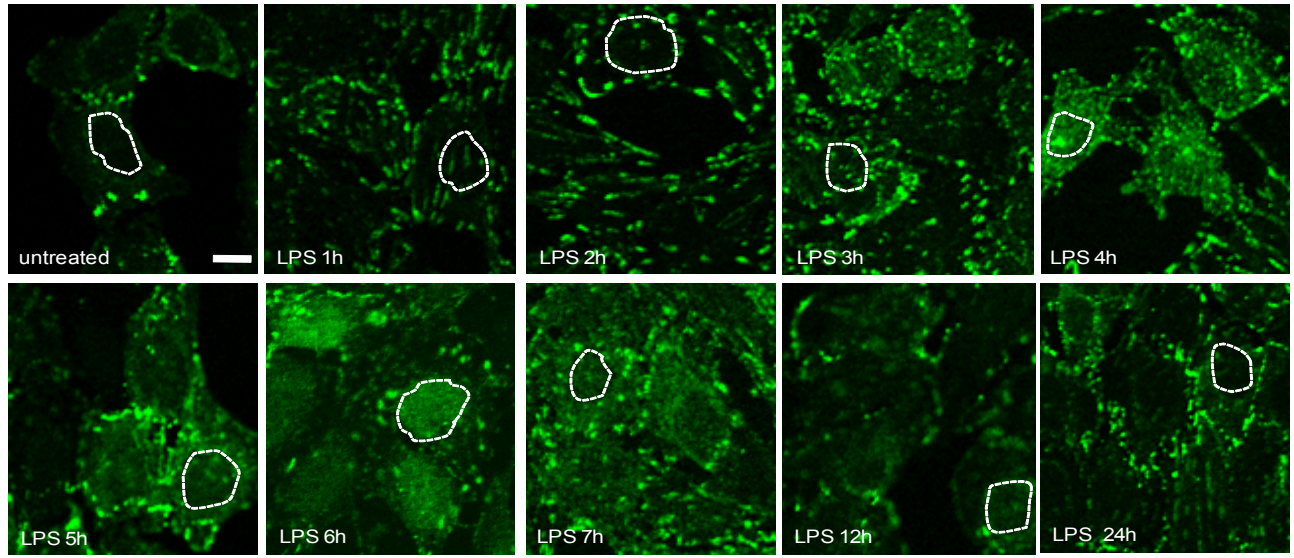
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Figure S3.

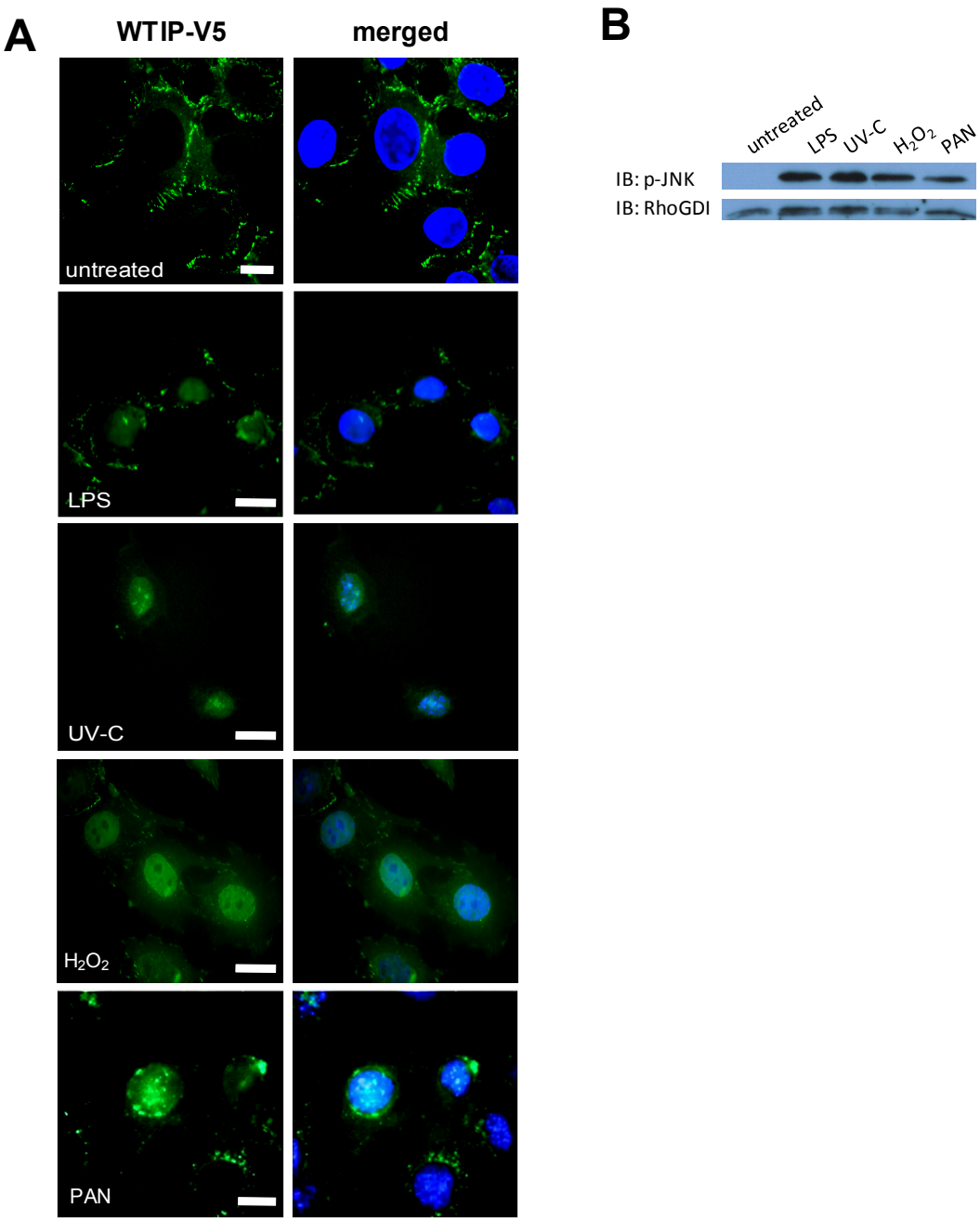
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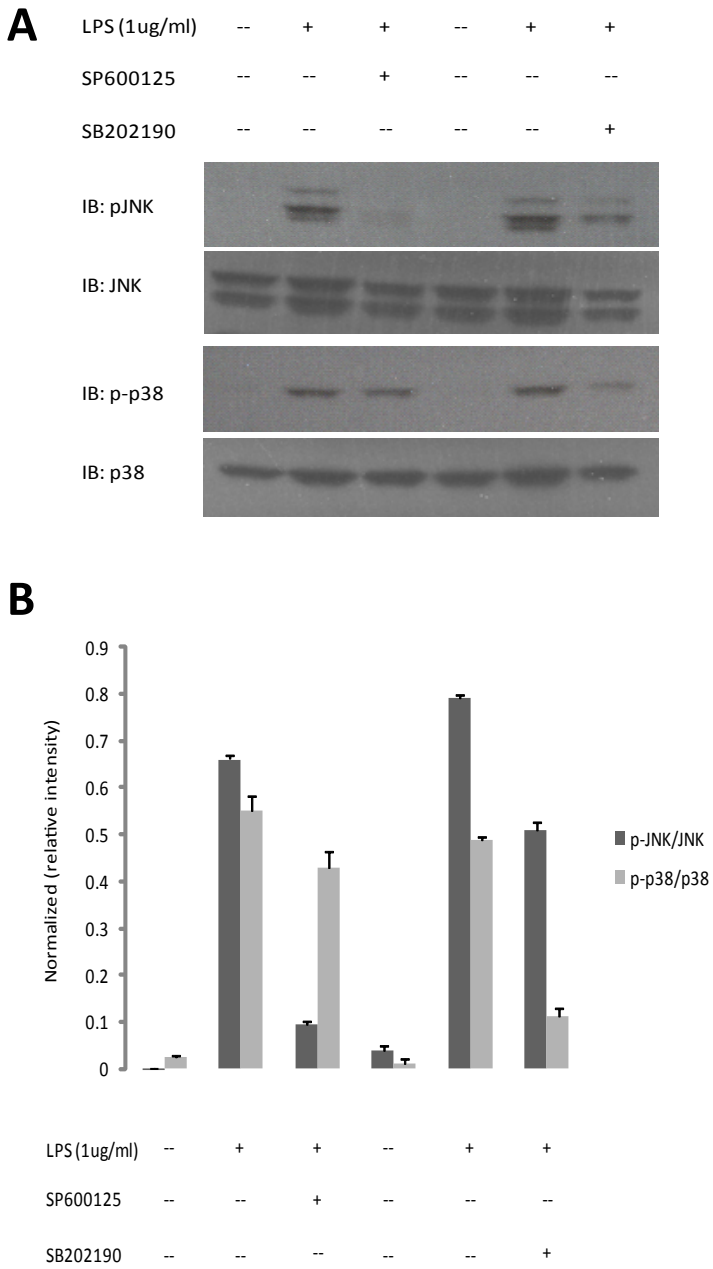
Figure S4.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Normalized (relative intensity)
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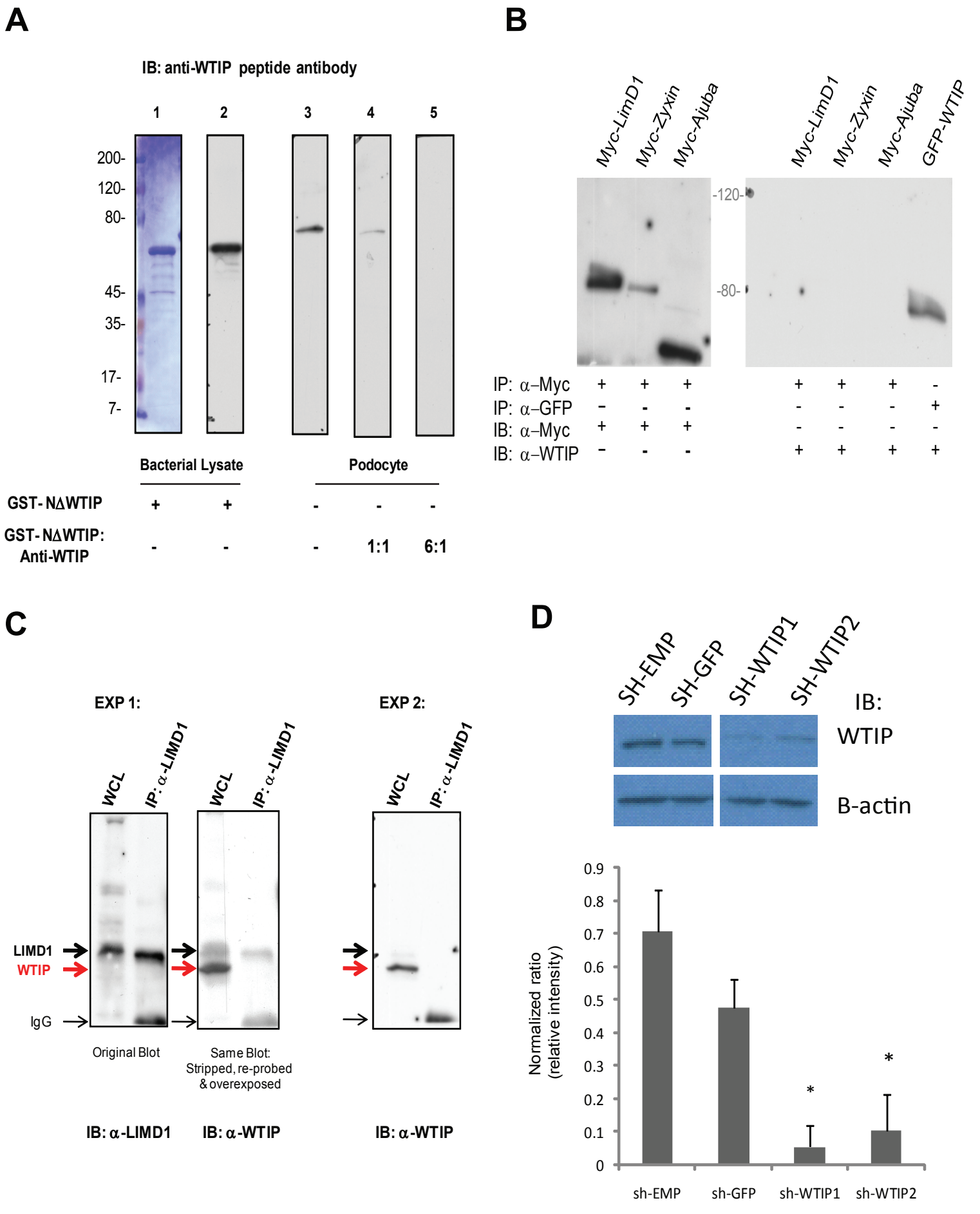
SB202190 -- -- -- -- -- +

Figure S5.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Bacterial Lysate
Podocyte
GST-NΔWTIP + + - - -
GST-NΔWTIP: Anti-WTIP - - - 1:1 6:1
B
Myc-LimD1
Myc-Zyxin
Myc-Ajuba
Myc-LimD1
Myc-Zyxin
Myc-Ajuba
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SH-GFP
SH-WTIP1
SH-WTIP2
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WTIP
B-actin
Normalized ratio (relative intensity)
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0.7
0.6
0.5
0.4
0.3
0.2
0.1
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sh-EMP
sh-GFP
sh-WTIP1
sh-WTIP2