MATERIALS AND METHODS

Materials and Antibodies

Drugs: Bis-T-23 (Aberjona Laboratories, Inc., Woburn, MA) and Dynole (Abcam, Cambridge, MA) were prepared as 30 mM stock solutions in DMSO and stored frozen. Y-27632 and blebbistatin were purchased from Sigma-Aldrich (St. Luis, MO). DMSO purchased from Sigma-Aldrich (0.1 %) was used as a control vehicle in all experiments.

Antibodies: Mouse monoclonal anti-Dyn1/2 (Hudy 1, EMD Millipore, Billerica, MA); mouse monoclonal anti-Dyn1 (Enzo Life Sciences, Farmingdale, NY); mouse monoclonal anti-GAPDH, mouse monoclonal anti-gelsolin, and mouse monoclonal anti-zyxin (abcam); mouse monoclonal TRITC-labelled anti-biotin (Jackson ImmunoResearcg Laboratories, Inc, West Grove, PA); rabbit monoclonal anti-paxillin (StressGene, Victoria, Canada); mouse monoclonal anti-mDia-1 (BD Biosciences, Sparks MD), and mouse monoclonal anti-FAK (BD Biosciences); rabbit polyclonal anti-paxillin pY31, and rabbit polyclonal anti-FAK pY397 (Invitrogen, Carlsbad CA); mouse monoclonal anti-RhoA, rabbit polyclonal anti-Rac1, and rabbit polyclonal anti-Cdc42 (Santa Cruz Biotechnology, CA). Other materials: Rhodamine-phalloidin and Fluorescence-phalloidin were from Life Biotechnologies. All other reagents were of analytical reagent grade or better.

Cell culture and standard techniques

A conditionally immortalized mouse podocyte cell line was grown as described elsewhere.[1] HEK 293T cells were grown in DMEM containing 10% fetal bovine serum (Sigma-Aldrich) supplemented with antibiotic/antimycotic (all from Life Biotechnologies). Adenoviral infections of cultured cells were performed to express various dynamin proteins in podocytes as described in[2] or according to manufacturer's protocol (Life Technologies). Cells were pretreated with RhoA signaling inhibitor Y-27632 (10 μM) or bebbistatin (20 μM) for 30 min prior to treatment with Bis-T-23 (30 μM), dynole (10 μM)

or DMSO (0.1 %). The number of barbed ends in podocytes was examined as described in.[3] Biotin G-actin was reconstituted in buffer (1 mM Hepes, pH 7.5, 0.2 mM MgCl2, and 0.2 mM ATP) and was subsequently centrifuged at 150,000 g for 30 min to remove aggregates. Mouse podocytes serum-starved for 3 hours were first pretreated with Y-27632 (10 μM) for 30 min followed by Bis-T-23 (30 μM), Dynole (10 μM) or DMSO (0.1 %) for 10 minutes. Cells were permeabilized in the presence of 0.45 μM biotin G-actin for 45 sec, fixed and incubated with TRITC-conjugated anti-biotin antibody for 1 hr. To determine the number of cells with barbed ends or barbed ends per cell, cell images in randomly chosen fields were acquired using a Zeiss LSM 5 PASCAL laser scanning microscope (40x objective) (Carl Zeiss, Thornwood, NY), and image processing was performed using Zeiss LSM 5 Image Browser Version 4.2.0.121, Photoshop 7.0 (Adobe Systems, San Jose, CA) and Image J software (National Institutes of Health, Bethesda, MD). Data were further analyzed using GraphPad Prism 5 (version 5.04) for Windows (GraphPad Software, San Diego, CA) to perform statistical analysis using unpaired two-tailed t-tests. Based on this analysis *$p<0.05$, **$p<0.01$, ***$p<0.001$, and $p>0.05$ is considered not significant (n.s). Western blot analysis was performed as described before.[3]

Lentiviral Knockdown

shRNA lentiviral plasmids to down-regulate dynamin2,[3] gelsolin, RhoA or mDia-1 were obtained from Sigma-Aldrich. The plasmids were used to generate lentiviral transduction particles in HEK293T cells. A target set of clones with pLKO.1-puro as the lentiviral-based plasmid were used, and designated as below with sequences:

Dynamin2:

S3: 5’ CCGGGCCCTTGAGAAGAGGCTATATCTCGAGATATAGCCTCTTCTCA

AGGGCTTTTTG 3’

S4: 5’ CCGGGCCCGCATCAATCGTATCTTTCTCGAGAAGATACGATTGATGC
GGGCTTTTTG 3’

S5: 5’ CCGGCCTAGTGGACATGACAATGAACTCGAGTTCATTGTCATGTCCA
CTAGGTTTTTG 3’

Gelsolin:

G6: 5’ CCGGGCTACTTCAAGTCTGGACTTACTCGAGTAAGTCCAGACTTGAA
GTAGCTTTTTG 3’

G8: 5’ CCGGGACTTCTGCTAAGCGGTACATCTCGAGATGTACCGCTTAGCAG
AAGTCTTTTTG 3’

G9: 5’ CCGGCTGCAGTATGACCTCCACTATCTCGAGATAGTGGAGGTCATAC
TGCAGTTTTTG 3’

RhoA

G1: 5’ CCGGCCCAGACTAGATGTAGTATTTCTCGAGAAATACTACATCTAGTC
TGGGTTTTTG 3’

G2: 5’ CCGGGTCAAGCATTTCTGTCCAAATCTCGAGATTTGGACAGAAATGC
TTGACTTTTTG 3’

mDia-1:

F1: 5’ CCGGGCAGGCTCAGTTTGAACTCAACTCGAGTTGAGTTCAAACTGAG
CCTGCTTTTTG 3’

F2: 5’ CCGGCCACAAGAACAATGCAAAGTTCTCGAGAACTTTGCATTGTTCTT
GTGGTTTTTG 3’

F3: 5’ CCGGGCGATCTCAAACTGTGGGAATCTCGAGATTCCCACAGTTTGAG
ATCGCTTTTTG 3’

F4: 5’ CCGGCCACAACCAAATGTGTCTGAACTCGAGTTCAGACACATTTGGTT

GTGGTTTTTG 3’

F5: 5’ CCGGCCACAACCAAATGTGTCTGAACTCGAGTTCAGACACATTTGGTT

GTGGTTTTTG 3’

Lentiviral knockdown of gelsolin, RhoA and mDia-1 was performed in differentiated mouse podocytes as per the protocol from the RNAi Consortium. Briefly, mouse podocytes were allowed to differentiate in 6 well dishes. 10 days into differentiation the media was replaced with fresh media containing 8 μg/ml polybrene followed by the addition of lentiviruses. The cells were incubated with the virus for 24 hours, after which the medium was replaced with fresh growth medium with 1.25 μg/ml puromycin. Selection was performed for 3 days, after which cells were harvested to assay for knockdown efficiency using qPCR and Western Blot.

High-throughput fluorescence microscopy

Fully-differentiated podocytes were cultured in 96-well multi-well optical plates (PerkinElmer, Boston, MA) and treated with various amounts of Bis-T-23 for 30 min. Subsequently, the cells were fixed by adding a solution of paraformaldehyde (4%) and sucrose (2%) for 1 h at room temperature followed by permeabilization with 0.3% Triton X-100 in PBS for 10 min at RT. Then F-actin was visualized by labeling with Alexa Fluor 594 phalloidin (Life Technologies). A nuclear/cytoplasmic stain, HCS CellMask Blue (Life Technologies), was also used to demarcate the boundaries of individual cells for quantitative analysis using high-content screening systems. The cells in 96-well optical plates were imaged using the Opera LX High-content confocal imaging system (PerkinElmer) with appropriate filters. The image analysis performed for these images involved the quantitation of a variety of cellular parameters. Assays were performed in five replicate wells.

Image analysis: The images were analyzed using the Columbus 2.3.1 high-content screening (HT screening) image data storage and analysis system (PerkinElmer). The CellMask Blue stained nuclei were detected using the "Find Nuclei" analysis module with method C and a common threshold of 0.85 and an area greater than 190 μm^2. The cytoplasm was detected using the "Find Cytoplasm" analysis module with method D with an individual threshold of 0.20. A lower threshold for total cell phalloidin intensity was set at 300 and then F-actin fibers were sectioned using the "Find Spots" analysis module with method B with detection sensitivity and splitting coefficients of 0.45 and 0.46, respectively. Approximately 1500 cells per well were analyzed using the output parameter "Relative spot to background intensity – mean per well."

Statistical analysis: The data were analyzed using GraphPad Prism and Microsoft Excel with the advanced analysis add-on and were compared using student's t-test. P-values less than 0.05 were considered significant.

Small G-protein activation assay

The activation of small G-proteins was determined using the G-LISA Activation Assay Biochem kit (BK124, 127 and 128 for RhoA, Rac1, and Cdc42 respectively) from Cytoskeleton according to the manufacturer's instructions. Briefly, cultured mouse podocytes that had been serum-starved for 24 hr were treated with Bis-T-23 (30 μM) or DMSO (0.1 %) as a control for 30 min. Parallel controls were stimulated with serum (10%) or EGF (100 ng/ml) for 5 min. Cell lysates were equalized and added to a 96-well plate pre-coated with Rho or Rac/Cdc42-GTP–binding proteins. After incubation, free inactive small G-proteins were removed by washing, and active small G-proteins attached to the wells were detected by specific primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase. The signal was measured

immediately after addition of the horseradish peroxidase detection reagent at 490 nm using a SpectraMax M2E (Molecular Devices, Sunnyvale, CA).

Actin and paxillin staining and quantification

Cells were stained with anti-paxillin antibody and rhodamine-phalloidin. Images were captured with a Zeiss LSM 5 PASCAL laser scanning microscope and a 40x objective. For quantification of actin, cells were imaged with above microscope using a fixed exposure for phalloidin, and the intensity of the actin staining was measured by Image J (v1.47m) software. Total fluorescence from control (DMSO-treated or wild-type Dyn1 infected) cells and mutant Dyn1-infected and/or drug-treated cells was analyzed separately, with staining intensity normalized to the control cells. The experiment was repeated at least three times. The number and size of focal adhesions were determined by integrated morphometry analysis performed using Image J (v1.47m) on thresholded images to select classified objects of a size range of ≥ 10 pixel^2 as focal adhesions, based on anti-paxillin staining. The analyzed particles command was used to measure number and size of focal adhesions. In case of quantification in Figure 2, F-actin and focal adhesions in cell body were selected and the intensity was measured. When indicated, data were further analyzed using GraphPad Prism (v 4.03) for Windows to perform statistical analysis using two-tailed unpaired t-tests. Based on this analysis, *P<0.05, **P<0.01, ***P<0.001 and P>0.05 was considered not significant (n.s.).

Electron microscopy of podocyte cytoskeletons

Experiments were performed as described elsewhere.[3] Briefly, podocytes were grown on glass coverslips 5 mm in diameter and cytoskeletons were revealed by treating the cells with 0.75% Triton X-100 in PHEM buffer (60 mM piperazine diethanesulfonic acid, 25 mM HEPES, 10 mM ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic

acid, 2 mM $MgCl_2$, pH. 6.9) containing 1 µM phallacidin. Cytoskeleton was washed in PHEM and fixed in 0.5% glutaraldehyde in PHEM for 10 min. Coverslips containing the cytoskeleton were fixed, blocked and subsequently stained. Dynamin antigenic sites were stained using a combination of two monoclonal anti-dynamin antibodies. Anti-mouse IgG-coated gold (10 nm) were used to detect dynamin. Samples stained with only secondary antibodies were used as control.

REFERENCES:

1. Mundel, P. *et al.* Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines. *Exp Cell Res* **236**, 248-58 (1997).
2. Sever, S. *et al.* Proteolytic processing of dynamin by cytoplasmic cathepsin L is a mechanism for proteinuric kidney disease. *J Clin Invest* **117**, 2095-104 (2007).
3. Gu, C. *et al.* Direct dynamin-actin interactions regulate the actin cytoskeleton. *EMBO J* **29**, 3593-606 (2010).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Bis-T-23 stimulates focal adhesion maturation in podocytes.

(**A, B**) Wild-type mouse podocytes were treated with 30 μM Bis-T-23 or 0.1% of DMSO (vehicle control) for 30 min. Immunofluorescence co-staining of phosphorylated paxillin and zyxin (A) or phosphorylated paxillin and dynamin (B) was performed with anti-paxillin pY31 antibody, anti-zyxin antibody or anti-dynamin antibody. Scale bar, 20 μm (A). (**C**) Bar graphs depicting the relative intensity of indicated proteins at focal adhesions (FA). Data represent measurements of >150 focal adhesions from 10 cells for each condition and are plotted as mean ± S.D. Phosphorylated paxillin:P-Pax. ***p<0.001, n.s., not significant. (**D**) Bar graphs depicting distribution of focal adhesions based on their size. Data represent measurements of >50 cells and are plotted as mean ± S.D. (n=3). **p<0.01, ***p<0.001, n.s., not significant. (**E**) Electron micrographs of dynamin antigenic sites visualized using monoclonal anti-dynamin antibody followed by a secondary antibody labeled with 10 nm gold particles. Red circles indicate dynamin-rich centers. (**F**) Bar graph depicting the number of dynamin-rich centers in DMSO (1%) or Bis-T-23 (30 μM) treated podocytes. 20 images, each covering 3 X 4 μm space, were analyzed per condition. The data are plotted as mean ± S.D (n=3).

Supplementary Figure 2. The effect of Bis-T-23-driven Dyn^{OLIGO} on actin cytoskeleton is dependent on its actin binding and oligomerization property.

(**A**) Schematic diagram showing domain structure of dynamin and the three distinct dynamin mutants used in this study. PH: (Pleckstrin-Homology), GED (GTPase Effector Domain), and PRD (Proline/arginine-Rich Domain). (**B**) Western blot analysis showing the expression levels of dynamin proteins in cells. (**C**) Fluorescence microscopy of cultured podocytes expressing different dynamin mutants: $Dyn1^{E/K}$ (increased actin binding), $Dyn1^{K/E}$ (decreased actin binding), $Dyn1^{I690K}$ (incompetent oligomerization). Where indicated, cells were treated with 30 μM Bis-T-23 for 30 min. FAs and F-actin were visualized with anti-paxillin antibody and rhodamine-

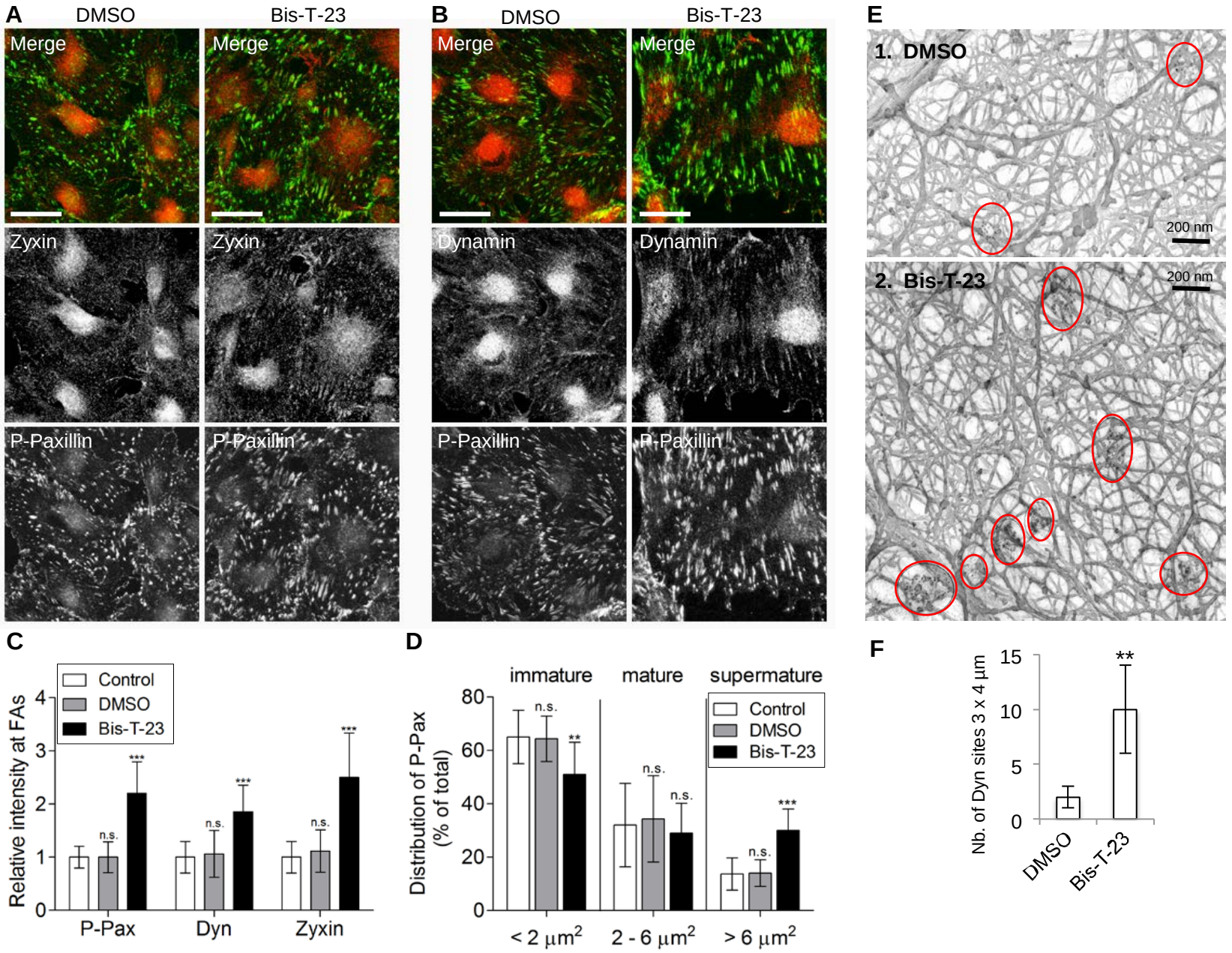
phalloidin respectively. Scale bar represents 20 µm. (**D**, **E**) Bar graphs depicting quantitative analysis of the number of FAs (D) and F-actin (E) per cell from experiments such as those in (C). The data represent measurements of > 50 cells and are plotted as mean ± SD (n=3). ***p < 0.001, n.s.:not significant.

Supplementary Figure 3. Bis-T-23-induced Dyn^{OLIGO} is capable of regulating actin cytoskeleton independently of RhoA signaling.

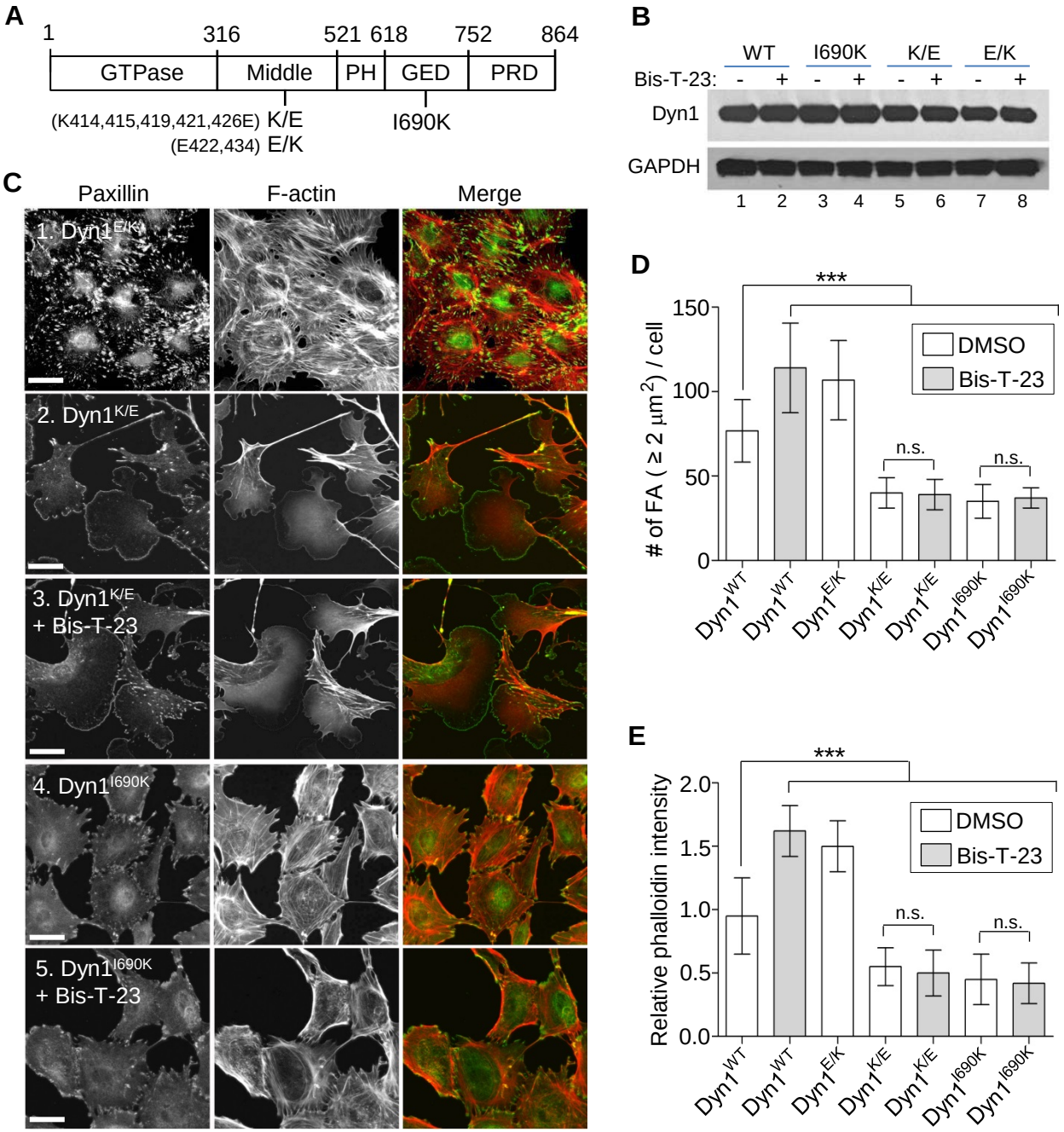
(**A**, **C**) Levels of mRNA for RhoA (A) and mDia-1 (C) determined by RT-PCR using two different primers (Primer 1 and Primer 2). Wild type (WT), control podocytes not infected with lentiviruses. Scr, cells infected with lentiviruses expressing a scrambled oligo. In case of RhoA, we used two different shRNA constructs named G1 and G2. In case of mDial-1, we used five different shRNA constructs named F1-F5. (**B, D**) Western blot analysis of protein levels for RhoA (B, lanes 7, 8) and mDia-1 (D, lanes 6-8) in cell extracts isolated from podocytes infected with lentiviruses expressing different shRNA constructs. Lanes 1-6 (B) and 1-5 in (D) show indicated volumes of cell extracts isolated from wild type cells not infected with lentiviruses. (**E**) Free barbed ends can be formed in the presence of ROCK inhibitor Y-27632 and Bis-T-23TM (30 µM). Podocytes were first incubated in the presence or absence of Y-27632 (10 µM for 30 min) to inhibit ROCK signaling. Subsequently, DMSO (0.1%) or Bis-T-23TM (30 µM) were added for 10 min. Cells were permeabilized in the presence of rhodamine labeled G-actin (0.45 µM, red) for 45 sec, fixed and total F-actin was labeled using FITC phalloidin (green). Scale bars are 10 µm. (**F, G**) Bar graphs depicting % of cells with free barbed ends (F) and the number of barbed ends per cell (G). The data are measurements of >50 cells as shown in (E) and are plotted as mean ± S.D. *p<0.05, **p<0.01, ***p<0.001, n.s.:not significant. (**H**) Western blot analysis of the amount of p-paxillin in podocytes in the presence of DMSO (0.1%) or Bis-T-23 (30 µM) after the treatment of ROCK inhibitor Y-27632 (10 µM).

Supplementary Figure 4. Bis-T-23-driven Dyn^{OLIGO} induces actin polymerization by generating free barbed ends in podocytes.

(**A**) Levels of Gsn mRNAs determined by RT-PCR. G6, G8 and G9, podocytes infected with three different shRNAs designed to downregulate Gsn. WT, control podocytes not infected with lentiviruses. Scr, cells infected with lentiviruses expressing a scrambled oligo shRNA. (**B**) Western blot analysis of gelsolin levels in podocytes infected with lentiviruses expressing shRNA constructs as indicated in figure (A). (**C**) Fluorescence microscopy of undifferentiated or fully differentiated podocytes expressing dynamin or treated with Bis-T-23. Where indicated, cells were treated with 30 μM Bis-T-23 for 30 min. FAs and F-actin were visualized with anti-paxillin antibodies and rhodamine-phalloidin respectively. Scale bar represents 10 μm. (**D**, **E**) Bar graphs depicting quantitative analysis of the number of FAs (D) and F-actin (E) per cell from experiments such as those in (C). The data represent measurements of > 50 cells and are plotted as mean ± SD (n=3). $^{**}p<0.01$, $^{***}p < 0.001$, n.s.:not significant.



Supplementary Figure 1. Gu et al.,



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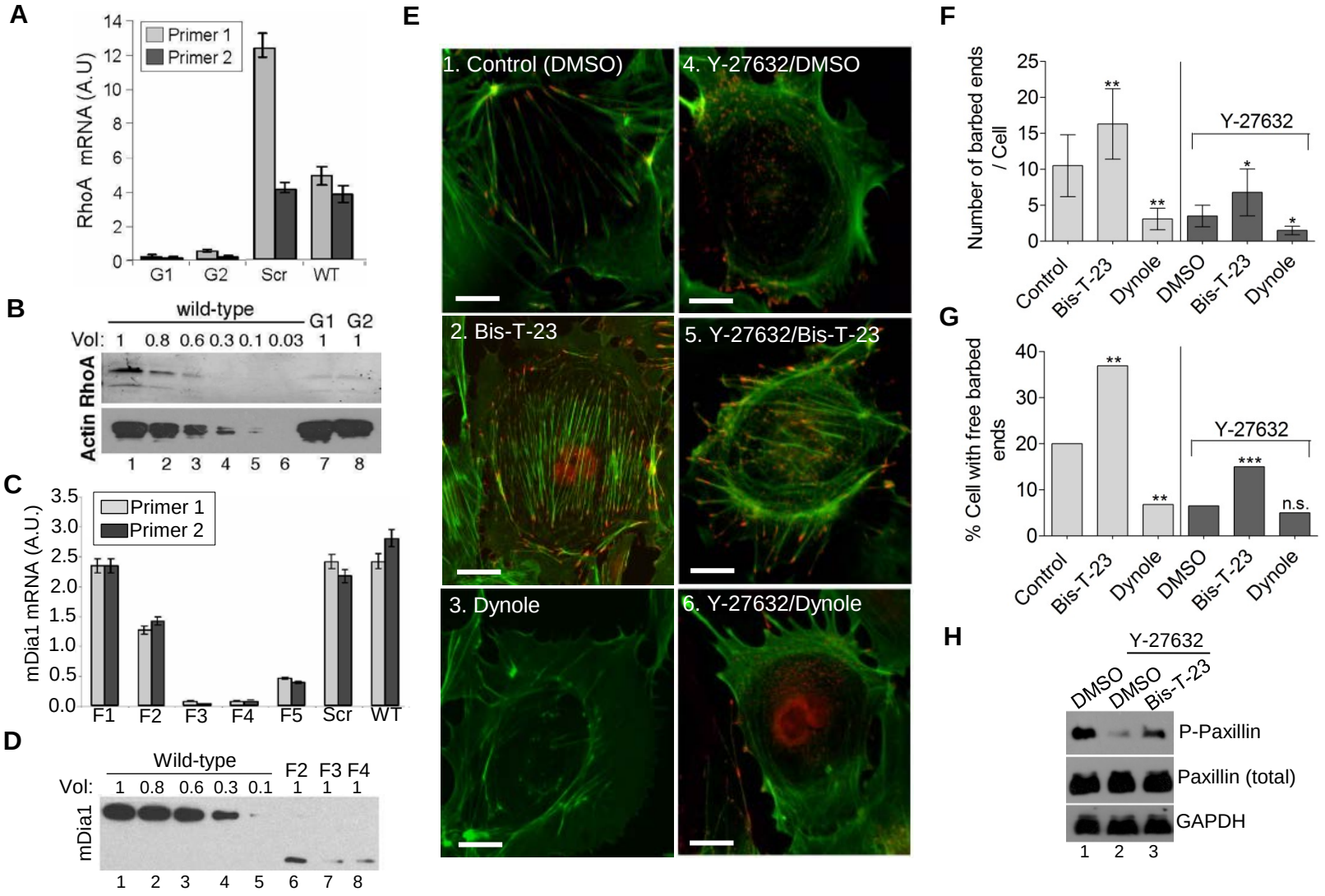

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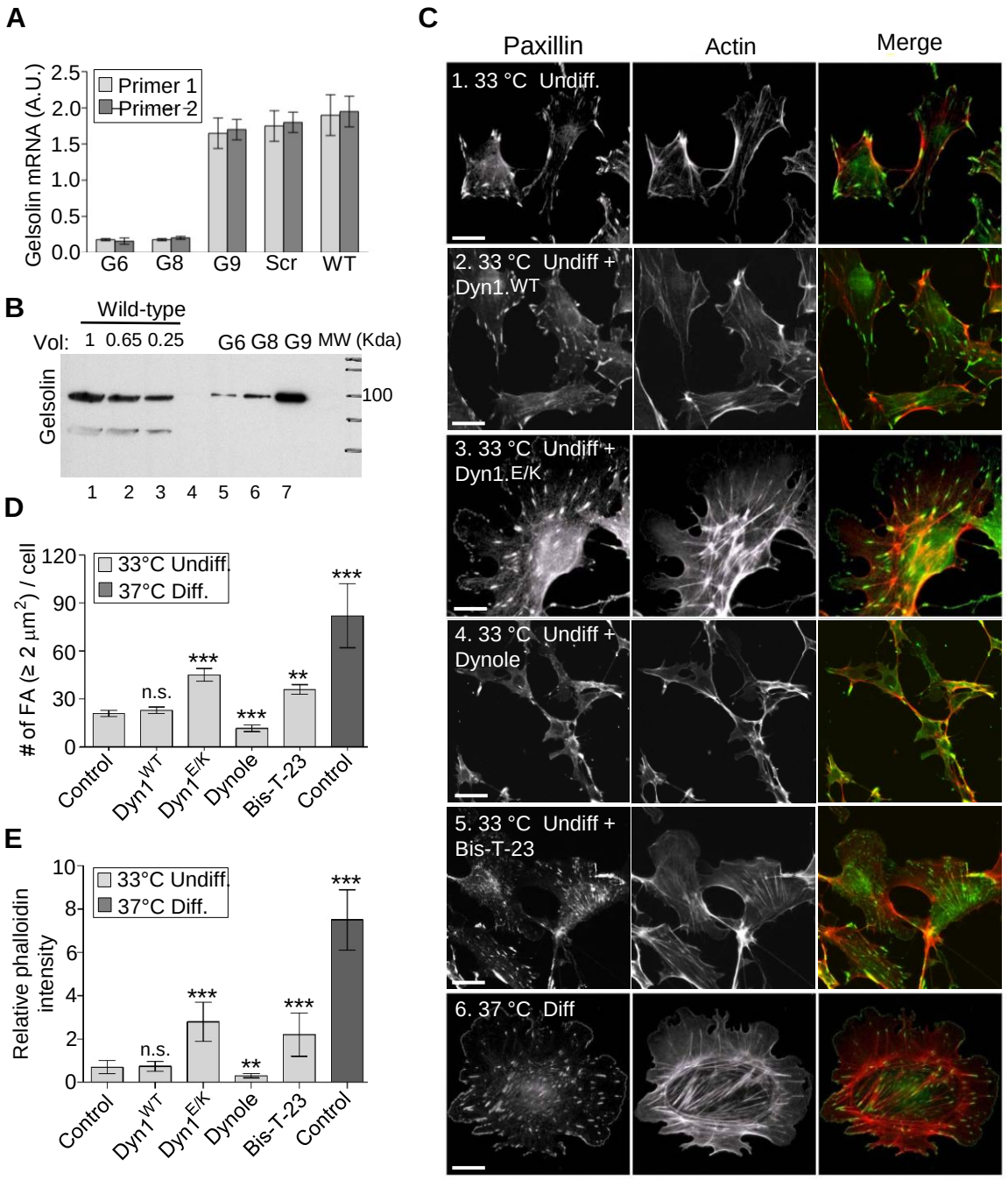

Supplementary Figure 4. Gu et al.,