

Supplementary Methods

Antibodies

The following antibodies were used: mouse monoclonal anti-Ezrin (Invitrogen, #357300), mouse monoclonal anti-ZO-1 (Invitrogen, #339100), mouse monoclonal anti-E-Cadherin (BD Biosciences #610181), mouse monoclonal anti- β -catenin (Sigma, #C7082), rabbit polyclonal anti-phospho- β -catenin (Ser33/37/Thr41) (Cell Signalling, #9561), mouse monoclonal anti-gp135 (a gift from C.Yeamann at University of Iowa, Iowa city, IA, and G.Ojakian at State University of New York Downstate Medical Center, Brooklyn, NY), mouse monoclonal anti- α -tubulin (Sigma, #T6074), mouse monoclonal anti-TfR (Invitrogen, #136800), mouse monoclonal anti-Golgin 97 (Invitrogen, #A21270), Alexa Fluor[®] 488 protein A conjugate (Molecular Probes, P11047), Alexa Fluor[®] 594 protein A conjugate (Molecular Probes, P11051), Alexa Fluor[®] 647 protein A conjugate (Molecular Probes, P21462), cell-permeant Hoechst DNA stain (Invitrogen), Tf conjugated to Alexa Fluor 594 (Invitrogen), goat anti-mouse AffiniPure F(ab')₂ fragments (Jackson ImmunoResearch Laboratories, Inc.), IRDye 800CW donkey anti-rabbit IgG (Li-Cor, 926-32213), and IRDye 680 donkey anti-mouse IgG (Li-Cor, 926-32222). Rabbit polyclonal anti-SNX18 and anti-cingulin antibodies were prepared as described previously [1] using recombinant purified SNX18 or cingulin. The antibodies were affinity purified using recombinant SNX18 or cingulin conjugated to Affigel (Bio-Rad Laboratories) and eluted with 0.1 M glycine buffer, pH 2.5. Rabbit anti-FIP5 and anti-FIP1 polyclonal antibodies have been described previously [1-3]. Rabbit polyclonal anti-pFIP5-T276 antibody was produced by 21st Century Biochemicals.

Generation and purification of adenoviral expression constructs

The phospho-silent mutant FIP5-T276A and the phospho-mimic mutant FIP5-T276D were generated using QuickChange[®] II XL Site-Directed Mutagenesis Kit (Stratagene, #200522) according to the manufacturer's instructions. Briefly, oligonucleotides containing mutated codons flanked by wild-type FIP5 sequences were used in PCR (used in PCR sounds wrong) to introduce the point mutation into the wild-type FIP5-GFP construct. shRNA-resistant FIP5-GFP adenoviral constructs and recombinant adenovirus were generated using the AdEasy system [6]. In brief, each gene was cloned into pShuttle-CMV, and the resultant clones were linearized with PmeI and used to transform E. coli BJ5183 cells carrying the viral DNA plasmid pAdEasy-1. Recombinant plasmids were digested with PacI to expose the inverted terminal repeats, and 8 µg of each construct were used to transfect, by calcium phosphate co-precipitation, 6-cm dishes of 50% confluent HEK 293 cells modified to express adenovirus preterminal protein, DNA polymerase, and DNA-binding protein. The media was aspirated and replaced after 24 h, and the cells were incubated for 10 d, until many plaques had formed. Virus was released by repeated freeze/thaw cycles and amplified by the addition of the adenoviral vector to fifty 10 cm dishes of HEK 293 cells, which were incubated for 48 h. Virus harvesting and purification were conducted as described previously [7]. In brief, the cells were harvested by centrifugation, and the virus was released by three freeze/thaw cycles followed by centrifugation to pellet the cell debris. Two rounds of virus back-extraction were performed on the cell pellet. The supernatants were combined and purified via centrifugation on a cesium chloride step gradient of 1 ml of 1.4 g/c² and 2 ml of 1.25 g/c² CsCl in PBS using an SW41 rotor centrifuged at 36,000 rpm. The virus banded at the interface of the CsCl steps, and was collected by side puncture with a syringe. The virus

was next mixed with 1.35 g/c² CsCl in PBS and centrifuged overnight at 65,000 rpm in an NVT100 rotor, and again collected by syringe side puncture. The resulting purified virion-containing solution was dialyzed four times for 2 h each at 4° against a modified previously published buffer containing 10 mM Tris, 10 mM His, 75 mM NaCl, 1 mM MgCl₂, 100 μM EDTA, 0.5% vol/vol EtOH, pH 7.4, and 50% vol/vol glycerol [8]. Virus particle concentrations were determined by OD₂₆₀ spectrophotometry, with one OD₂₆₀ unit equal to 10¹² particles.

GSK-3 inhibition assays

MDCK-MIIR cells were treated with either 1.5 μM GSK-3 Inh. or the equivalent volume of DMSO for 24 h at 37°C. Cells were then lysed in PBS containing 1% Triton X-100 and Phosphatase Inhibitor Cocktail Set II (EMD Millipore, #524625), and 60 μg of lysates were separated by SDS-PAGE for Western Blotting. For imaging, cells were plated either on Transwell filters or embedded into Matrigel before addition of the GSK-3 Inh.. Cells were then incubated for 24 h, fixed and processed for immunofluorescence analysis.

HeLa cell synchronization and mitotic shake off assays

Semi-confluent HeLa cells were incubated with thymidine for 16 h to arrest them at S-phase. Cells were then washed and incubated with serum-supplemented media for 8 h to allow cells to progress to pro-metaphase. Cells were then incubated with 100 μg/ml of nocodazole for another 6 h to arrest them in metaphase. Metaphase cells were isolated by gently tapping tissue culture dish to dislodge weakly-attached mitotic cells. Cells were then washed to release them from nocodazole block and incubated in serum-supplemented media for varying amounts of time. Cells from different mitotic stages

were then harvested and levels of FIP5, pFIP5-T276, GSK-3, TfR (loading control) and cyclin B (synchronization control) analyzed by western blotting.

***In vitro* phosphorylation assay**

Purified recombinant 6His-FIP5 (2 μ g), GSK-3 β (NEB, P6040S) (0.5 μ L), ATP (200 μ M) and DMSO (the same volume as GSK-3 Inh.) or GSK-3 Inh. (1.5 μ M) were used in reactions of 20 μ L as indicated in manufacturers protocol (NEB, P6040S). Reactions proceeded at 30°C for 30 min and were terminated by adding SDS-PAGE loading buffer. Samples were then analyzed by western blotting with anti-FIP5 and anti-pFIP5-T276 antibodies.

Peptide dot blot assay

A dilution series of peptides with or without phosphorylation at FIP5-T276 were bound to Hybond ECL nitrocellulose membranes (GE Healthcare, RPN3032D). Membranes were allowed to dry at RT overnight and blocked for 30 min in 5% milk. Membranes were then incubated with pre-immune bleed or affinity purified anti-pFIP5 antibody in milk for 2 h at RT. After washing, membranes were incubated with secondary antibody for 1 h at RT, washed, and scanned for blotting. Peptide sequences:

C-Ahx-GPGTELL[pT]HSPSRSS-amide

Acetyl-GPGTELL[pT]HSPSRSS-Ahx-C-amide

C-Ahx-GPGTELLTHSPSRSS-amide

Supplementary References

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