METHODS

Cell culture, antibodies, and reagents

The following cells including 293T, Caco2, DLD1, SW480, and WT and rictor$^{-/-}$ mouse embryonic fibroblast cells were utilized in this study. All cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin. The shRNAs for mouse PKCζ gene was constructed in pLKO.1-puro vector and purchased from Sigma-Aldrich. Two shRNA constructs were used to generate stable PKCζ knockdown MEF cells, and the targeting sequences were as the following: 5'-GAAGTGCTCATCATTCATGTT -3' (clone #1) and 5'- CAGATGATGAGGACGTCATAA -3' (clone #2). The lentivirus-mediated delivery of shRNA and selection for stable knockdown cells were carried out as previously described (Liu et al, 2009). Stable raptor, rictor, and mTOR knockdown colon cancer cell lines have been described in our previous studies (Gulhati et al, 2011; Liu et al, 2011).

The rictor and raptor antibodies used for immunoprecipitation were obtained from Bethyl Laboratories. The phospho-Akt (Ser473), phospho-PKCζ (T410), phospho-S6 (Ser235/236), S6, mTOR, rictor, raptor, and Cdc42 antibodies used for Western blotting were from Cell Signaling. The phospho-PKCζ (T560) (also called PKCι[pT555]/PKCλ[pT563] antibody) was obtained from Invitrogen. The PKCζ (including the mouse monoclonal and rabbit polyclonal), PKCι/λ, Akt1/2/3 (mouse monoclonal antibody recognizes all three Akt isoforms), RhoA, and ubiquitin (P4D1) antibodies were purchased from Santa Cruz Biotechnology. The Rac1 antibody was from Millipore. The anti-γ tubulin and Flag monoclonal antibodies were from Sigma-Aldrich.

The expression plasmids of Flag-PKCζ (WT), Flag-PKCζ/T410A, and Flag-PKCζ/T410E originally developed by Dr. Alex Toker (Chou et al, 1998) were purchased from Addgene.

Additional PKCζ mutant plasmids including Flag-PKCζ/T560A and Flag-PKCζ/T560D were generated by site-directed mutagenesis (Stratagene QuikChang kit) using Flag-PKCζ as the template. The EGFP-tagged PKCζ/T560D expression construct was created by subcloning PKCζ/T560D into pEGFP-N1 vector (Clonetech). The Myc-tagged rictor (Myc-Rictor) expression construct was obtained from Addgene.

Ubiquitination and degradation of PKCζ

To examine the ubiquitination of PKCζ, 293T cells transfected with Flag-tagged WT or 560A mutant PKCζ expression constructs or WT and $Ric^{-/-}$ MEF cells were pre-treated with HSP90 inhibitor 17-AAG (1 μM) for overnight and subsequently treated with DMSO or MG-132 (20 μM) for 2 hours. Cell lysates were prepared in lysis buffer as described previously (Li et al, 2009) and subjected to immunoprecipitation with the anti-Flag agarose or the PKCζ rabbit polyclonal antibody coupled to protein A/G agarose. The immunoprecipitates were resolved by SDS-PAGE and the level of ubiquitination was detected using the ubiquitin monoclonal antibody. To determine the degradation time course of PKCζ, 293T cells transfected with Flag-tagged WT and mutant PKCζ were pretreated with 17-AAG (1 μM) and subsequently treated with cycloheximide (CHX, 20 μg/ml). Cell lysates were prepared at indicated time points in SDS sample buffer directly, and equal amounts of lysates were analyzed by immunoblotting.

Immunofluorescence staining

For PKCζ rescue experiments, $Ric^{-/-}$ MEF cells were transiently transfected with EGFP-PKCζ/560D expression plasmid. To detect the patterns of actin organization, MEF cells,

including WT, Ric$^{-/-}$, or Ric$^{-/-}$ transfected with EGFP-PKCζ/560D, were serum starved for 4 hours. The cells were subsequently seeded onto fibronectin coated glass coverslips and allowed to attached for 1 hour. Cells were then treated with EGF (10 ng/ml) for 15 minutes and fixed in 4% paraformaldehyde. F-Actin was visualized using Alexa594-conjugated phalloidin and as described previously (Larson et al, 2010). Images were taken using an Olympus FlowView FV1000 confocal laser scanning microscope (Li et al, 2011).

References

Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, Newton AC, Schaffhausen BS, Toker A (1998) Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Curr Biol* **8**(19)**:** 1069-1077

Gulhati P, Bowen KA, Liu J, Stevens PD, Rychahou PG, Chen M, Lee EY, Weiss HL, O'Connor KL, Gao T, Evers BM (2011) mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. *Cancer Res* **71**(9)**:** 3246-3256

Larson Y, Liu J, Stevens PD, Li X, Li J, Evers BM, Gao T (2010) Tuberous sclerosis complex 2 (TSC2) regulates cell migration and polarity through activation of CDC42 and RAC1. *J Biol Chem* **285**(32)**:** 24987-24998

Li X, Liu J, Gao T (2009) beta-TrCP-mediated ubiquitination and degradation of PHLPP1 are negatively regulated by Akt. *Mol Cell Biol* **29**(23)**:** 6192-6205

Li X, Yang H, Liu J, Schmidt MD, Gao T (2011) Scribble-mediated membrane targeting of PHLPP1 is required for its negative regulation of Akt. *Embo Rep* **12**(8)**:** 818-824

Liu J, Stevens PD, Gao T (2011) mTOR-dependent regulation of PHLPP expression controls the rapamycin sensitivity in cancer cells. *J Biol Chem* **286**(8)**:** 6510-6520

Liu J, Weiss HL, Rychahou P, Jackson LN, Evers BM, Gao T (2009) Loss of PHLPP expression in colon cancer: role in proliferation and tumorigenesis. *Oncogene* **28**(7)**:** 994-1004