Supplemental Information

PIPKIγ regulates β-catenin transcriptional activity in mesenchymal-like cells downstream of growth factor receptor activation

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Supplemental Experimental Procedures

Reagents

Polyclonal antibodies to PIPKIγ were created as previously described (1). Anti–HA was obtained from Covance; anti-EGFR(LA22), anti-GAPDH, anti-β-catenin, anti-actin and anti-phospho-EGFR(Y1173) were obtained from Millipore; anti-β-catenin was obtained from BD Transduction Laboratories; anti-phospho-β-catenin(Y86), anti–mouse and anit-rabbit Alexa Fluor 488 and 555 conjugates, and SlowFade reagent were obtained from Invitrogen; anti-phospho-β-catenin(S675), anti-phospho-β-catenin(S552), anti-phospho-β-catenin(S33/37), anti-LRP6, anti-phospho-LRP6, anti-c-met, anti-c-jun and anti-phospho-c-met(Y1234/1235) were obtained from Cell Signaling Technology; anti-phospho-β-catenin(Y654), anti-phospho-EGFR(Y1086), anti-HA, anti-Smad2/3 and anti-phospho-Smad2/3 were obtained from Abcam; anti-phospho-β-catenin(S45) was obtained from Biosource; anti-myc (9E10) was obtained from Santa Cruz Biotechnology; Doxycycline was obtained from Clontech; Wnt-3a and TGF-β were obtained from R&D Systems; EGF and HGF were obtained from Sigma; Horseradish peroxidase-conjugated anti-GST antibody was purchased from Amersham Biosciences and horseradish peroxidase-conjugated anti-T7 antibody was obtained from Novagen. Secondary horseradish-peroxidase-conjugated antibodies for Western blotting were obtained from Jackson Immunoresearch Laboratories. PIPKIγ and PIPKIα mammalian and bacterial expression vectors were described previously (2). pCMV-HA PIPKIγi4_^{KD}, PIPKIγ_i5^{KD} and PIPKIα^{KD} were generated using the QuikChange II Site-Directed Mutagenesis kit

(Stratagene). pCMV-myc containing the E-cadherin C-terminus was previously described (2).

Cell culture and transfection

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Hela TET-OFF cells stably expressing various PIPKIγ constructs have been described elsewhere (2). Hela and MDCK cells were obtained from ATCC. For growth factor stimulation experiments, cells were serum starved in DMEM+0.5% FBS for 12-24 h and then stimulated with the appropriate growth factor.

Adherent cells were transfected with Lipofectamine^{TM} 2000 (Invitrogen) or FUGENE$^{®}$ 6 transfection reagents according to the manufacturer's instructions; transient transfection conditions were optimized for maximum expression and minimal toxicity.

Lysate preparation, and immunoblotting

Whole-cell extracts were prepared in RIPA lysis buffer (20mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 2 mM EDTA, 1% [vol/vol] Nonidet P-40, 150 mM NaCl, 0.1% SDS, 50 mM NaF, supplemented with protease inhibitors). For immunoprecipitation experiments, lysates were prepared in a modified RIPA buffer (50mM Tris-HCl, pH 7.2, 0.5% sodium deoxycholate, 2 mM EDTA, 1% [vol/vol] Triton X-100, 50 mM NaCl, 0.1% SDS, supplemented with Complete Protease Inhibitor Mixture (Roche)). Lysates were sonicated, cleared of debris by centrifuation at 15,0000xg for 10 min at 4°C and protein concentrations calculated using the BCA (bicinchoninic acid) protein quantification assay (Bio-Rad Laboratories) according to the manufacturer's instructions. For all IPs, samples of equal protein concentration and volume were pre-cleared with normal mouse or rabbit IgG (Millipore) and Protein A/G sepharose beads (GE Healthcare). IPs were carried out overnight at 4°C followed by washing in modified RIPA buffer 5X. For immunoblot analysis, proteins were resolved by SDS-PAGE and transferred to Immobilon polyvinylidene fluoride filters (Millipore). Blots were incubated 30 min in PBS-T (PBS +

0.1% Tween-20) containing 5% nonfat dry milk or 3% BSA. The blots were incubated with primary antibody overnight at 4°C followed by incubation with HRP-conjugated secondary antibodies, and immunolabeled proteins were visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer).

Immunofluorescence and microscopy

HeLa or Hela TET-Off in DME + 10% FBS cells were grown on glass coverslips placed inside six-well plates 24 h prior to transfection. Cells growing on glass coverslips were washed in PBS and fixed in PBS plus 4% PFA for 30 min at room temperature or overnight at 4°C, permeabilized in PBS containing 0.2% Triton X-100, and incubated for 30 min in PBS containing 3% BSA (Jackson Immunoresearch Laboratories) and 2% normal goat serum (Calbiochem). Samples were incubated with primary antibodies for 1 h at room temperature. Incubation with fluorophore-conjugated secondary antibodies was performed at 37°C for 30 min. Signals were developed using either Alexa Fluor 488 goat anti–rabbit or anti–mouse IgG for 1 h at room temperature. Samples were mounted in SlowFade antifade reagent containing DAPI. Indirect immunofluorescence microscopy was performed on a Nikon Eclipse TE2000U instrument equipped with a Photometrics CoolSNAP CCD (charged coupled device) camera. Images were captured and further processed using MetaMorph (Molecular Devices) cellular imaging software. Images were exported to Photoshop CS2 (Adobe) for final processing and assembly.

GST affinity pull-down assays

Biochemical affinity precipitation assays to measure an association between β-catenin and PIPKIγ were performed essentially as described previously (3). In brief, constructs in pET28 and pET42 were transformed into BL21 (DE3) competent cells (Novagen). Proteins were expressed and purified using His-Bind Resin following manufacturer's instructions (Novagen) or using glutathione-Sepharose 4B Fast Flow as per the manufacturer's instructions (Amersham Biosciences). Equal amounts of purified

recombinant GST-tagged PIPKIγ and T7-tagged β-catenin splice variants were incubated with 50 ul 1:1 diluted glutathione-Sepharose 4B Fast Flow (Amersham Biosciences) in 500 ul of pulldown buffer (PBS, 0.5% NP-40, and 2mM DTT) overnight at 4^0C while rotating. Purified GST alone incubated with T7-tagged β-catenin splice variants was used as a negative control for non-specific protein binding. The beads were washed with 1 ml ice-cold pulldown buffer four times, resolved by SDS-PAGE, and analyzed via Western blot. All tubes, reagents, and rotors were prechilled on ice before use and all steps were performed in a cold room.

References

1. Ling K, Doughman RL, Firestone AJ, Bunce MW, Anderson RA. Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. Nature 2002;420(6911):89-93.

2. Ling K, Bairstow SF, Carbonara C, Turbin DA, Huntsman DG, Anderson RA. Type Igamma phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with mu 1B adaptin. J Cell Biol 2007;176(3):343-53.

3. Ling K, Doughman RL, Iyer VV, et al. Tyrosine phosphorylation of type Igamma phosphatidylinositol phosphate kinase by Src regulates an integrin-talin switch. J Cell Biol 2003;163(6):1339-49.

Figure S1 (relates to Fig. 2)

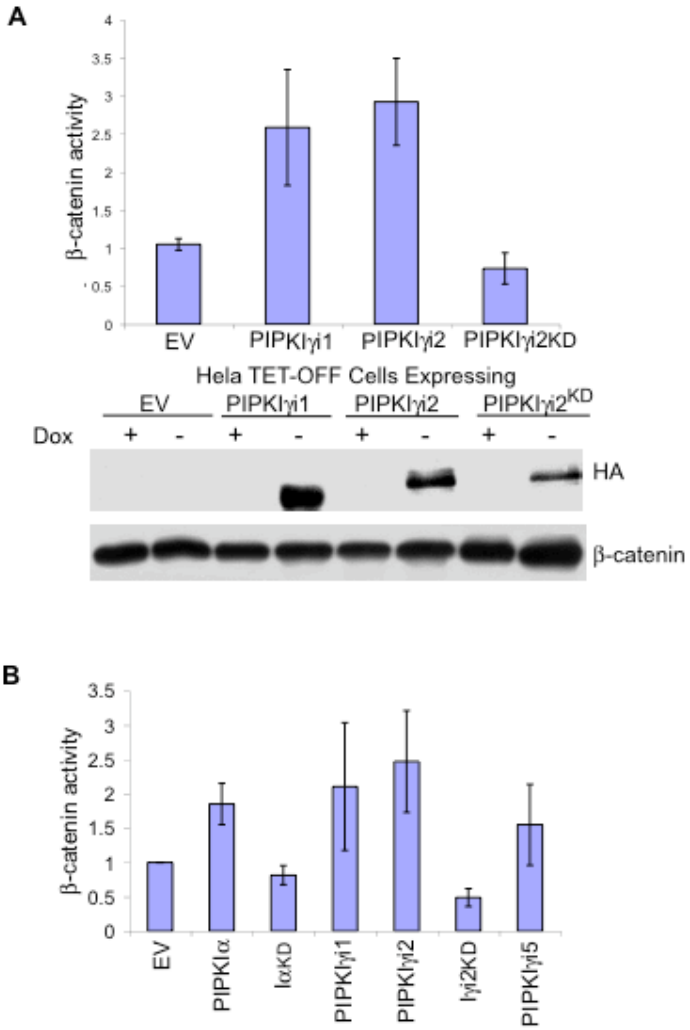

Figure S1. **PIPKIγ expression stimulates β-catenin transcriptional activity in Hela TET-OFF and HEK293T cells.** (A) Hela TET-OFF cells stably transfected with empty vector, PIPKIγ_i1, PIPKIγ_i2 or PIPKIγ_i2^{KD} were grown for 24 h in DMEM+10%FBS +/- doxycycline where indicated. Cells were transfected with the TOP-FLASH reporter construct and β-catenin activity quantified 24 h later. Western blotting using anti-HA confirmed the expression of the PIPKIγ isoform and anti-β-catenin monitored the effects of PIPKIγ expression on overall protein levels. (B) HEK293T cells were transiently transfected with the TOP-FLASH luciferase β-catenin reporter construct and with either empty vector (EV) or the HA-tagged type I PIPK shown and β-catenin activity assayed (n=3, error bars=std dev.).

Figure S2 (relates to figure 3)

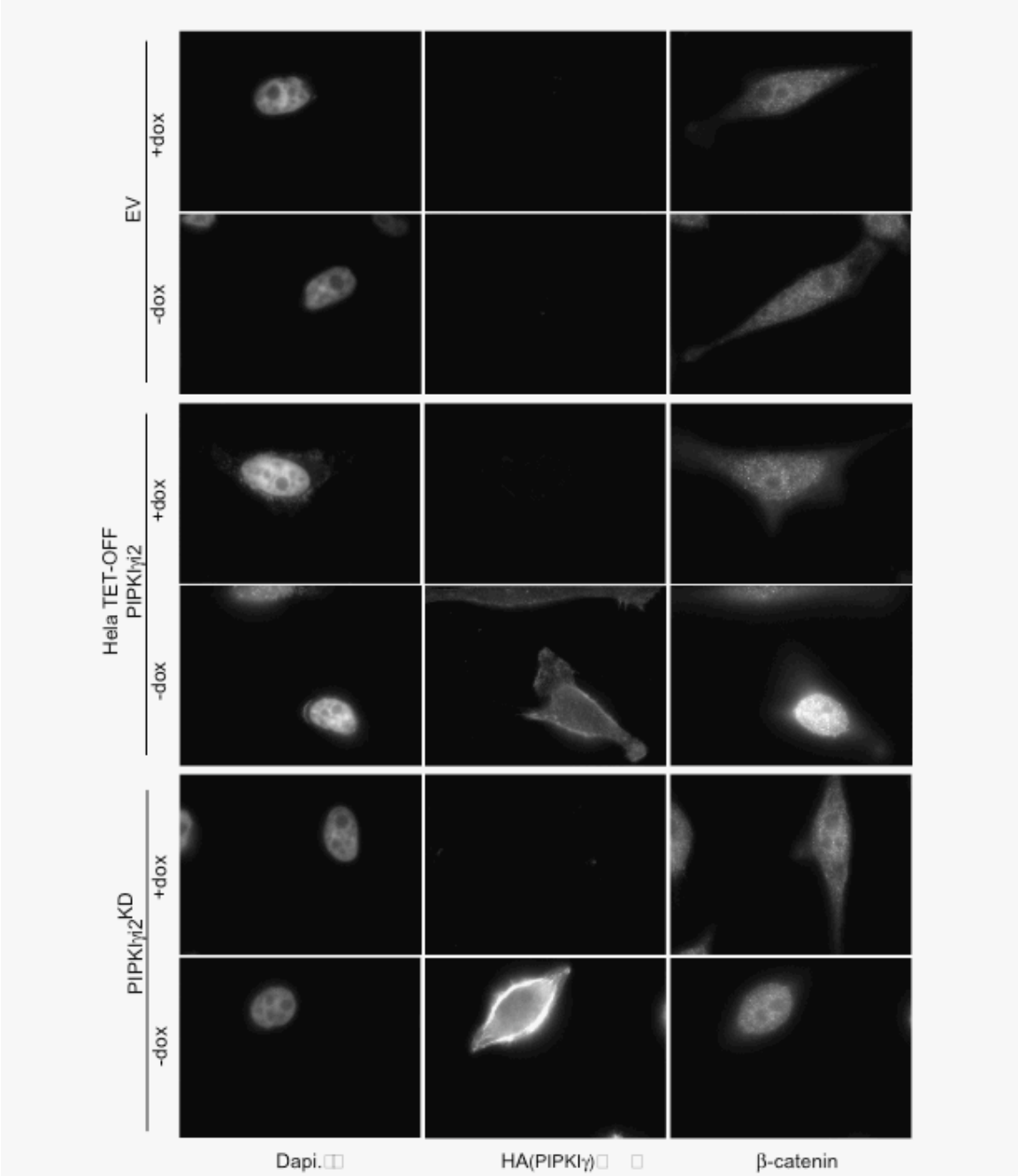

Figure S2. **PIPKIγ generation of PIP_2 increases the nuclear accumulation of β-catenin**. Hela TET-OFF cells stably expressing empty vector (EV) or HA-tagged PIPKIγ_i2 or PIPKIγ_i2^{KD} were grown on glass coverslips in DMEM+ 10%FBS +doxycycline. To initiate PIPKIγ_i2 expression, media was replaced with fresh media +/- dox. where indicated. 24 h later, cells were fixed and stained with dapi, anti-HA and anti-β-catenin. All images were taken with a 60X objective.

Figure S3 (relates to figure 5)

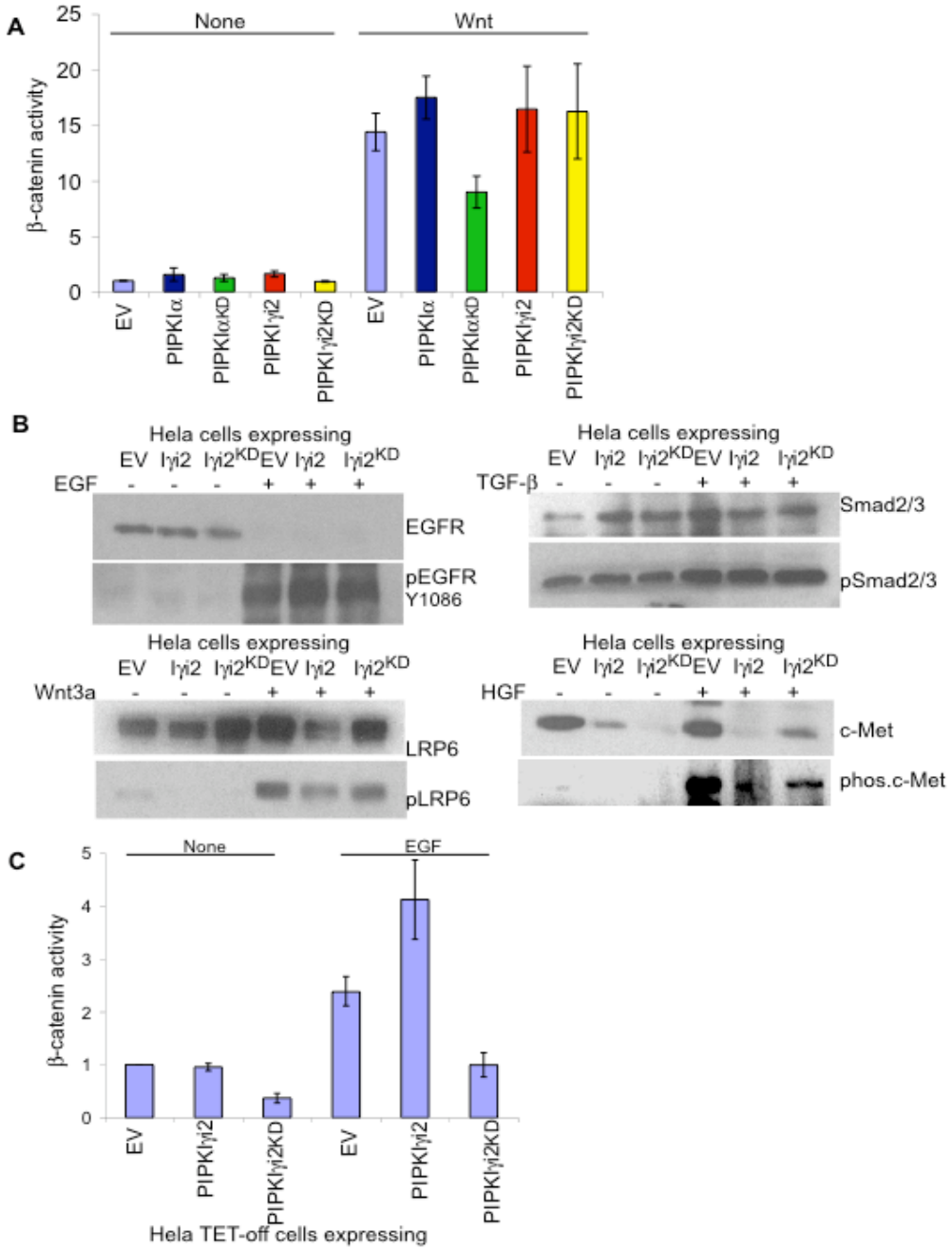

Figure S3. **Effect of PIPKIα or PIPKIγ expression on Wnt-dependent β-catenin activity.** β-catenin activity was measured in Hela cells transiently transfected with empty vector (EV) or the HA-tagged PIPKI shown. 24 h after transfection, cells were serum starved and then left untreated (none) or stimulated with 100ng/ml wnt-3a where indicated followed by cell lysis to measure β-catenin activity. (B) The efficacy of growth factor stimulation in transiently transfected Hela cells was monitored by western blotting using activation specific antibodies. Shown are representive blots of at least 3 experiments. (D) β-catenin activity was measured in Hela TET-OFF cells stably

transfected with empty vector, PIPKIγ_i2 or PIPKIγ_i2^{KD}. Cells were serum starved in DMEM+0.5%FBS without doxycycline. Cells were treated with 1nM EGF and β-catenin activity quantified 24 h later (n=3, error bars=std dev.).