Supplemental Methods

Cell Culture SKCO15 human colonic epithelial cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU of penicillin, 100 μg/ml streptomycin, 15 mM HEPES, and 1% nonessential amino acids (Cellgro; Mediatech, Herndon, VA). The cells were subcultured and harvested with 0.05% trypsin with EDTA in Hanks' balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO). Human embryonic kidney (HEK 293) JAM-A expressing cell lines, including the full length protein and a truncation mutant lacking the N-terminal Ig-like loop (DL1) were described previously (Mandell et al, 2004). Anti-JAM-A shRNA stable SKCO15 cell lines were produced by incorporation of linearized Tet-on vector ptTs-Neo, and pTMP (Thermo Scientific Lafayette, CO) expressing shRNA: TGGCATTGGGCAGTGT TACA**TTCAAGAGA**TGTAACACTGCCCAATGCCTTTTTGGAAAC (Konopka et al, 2007). Stable cells were cultured with puromycin (maintained in 0.5 ug/ml, selected in 2.5ug/ml) and neomycin (maintained in 100ug/ml, selected with 400ug/ml).

Immunoblots Monolayers of epithelial cells that were between 60 and 90% confluent were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer containing 20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.4. Lysis buffer was supplemented with protease and phosphatase inhibitor cocktails from Sigma-Aldrich (1:100 dilution). Protein concentrations in lysates were quantified by bicinchoninic acid assay. Lysates were cleared by centrifugation and immediately boiled in reducing SDS sample buffer. Distal colonic epithelial cell lysates were prepared after the serosa and external longitudinal

layer of the muscularis propria were stripped away. Isolated epithelial sheets were subsequently lysed in RIPA buffer. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblots were performed by standard methods. Each immunoblot shown is representative of at least three independent experiments.

Immunofluorescence (IF) Microscopy Cells grown on coverslips were fixed and permeabilized with either 100% methanol or ethanol at −20°C for 20 min, or PFA 2.0% wt/vol for 10 min followed by 0.5% vol/vol Triton X-100 for 5 min. Frozen tissue sections were fixed in 100% ethanol at –20°C for 20 min, and blocked in 1% BSA in HBSS+ for 1 h. Primary antibodies were diluted in blocking buffer and incubated with cells for 1 h at 25°C. The cells were washed in HBSS+ and then incubated in fluorescently labeled secondary antibodies for 45 min at room temperature. Labeled sections were then washed and mounted in Prolong Antifade Agent (Invitrogen, Carlsbad, CA). A laser scanning microscope (Carl Zeiss, Jena, Germany) was used to capture confocal fluorescence images.

Antibodies and Reagents The antibodies used in this study are as follows: phospho-β-catenin Ser552 was produced as described previously (He et al, 2007), rabbit anti-phospho-Thr 308, PTEN, pan-Akt and β-catenin (Cell Signaling Technology, Danvers, MA) goat-biotinylated polyclonal antibody against mouse JAM-A (R&D Systems, Minneapolis, MN), polyclonal rabbit anti phospho-PTEN Ser380/Thr382/382 (Santa Cruz Biotec, Santa Cruz, CA), polyclonal rabbit anti-JAM-A (Zymed Laboratories, South San Francisco, CA), monoclonal mouse anti-tubulin (Sigma-Aldrich, St Louis MO), polyclonal rabbit anti-actin (Sigma-Aldrich), monoclonal rabbit anti-JAM-A (Novus

Biologicals, Littleton, CO), rabbit anti-Caspase-3 (R&D Systems), ILK (Millipore, Billerica, MA), Secondary antibodies included, goat anti-rabbit-horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, West Grove, PA), and goat anti-mouse-HRP (Jackson ImmunoResearch Laboratories). Nuclei were visualized with Topro-3 iodide (Molecular Probes). PI3K inhibitor LY294002 (1.5μM) from Sigma-Aldrich. AKT inhibitors Triciribine, Akt Inhibitor X (3μM), and Akt-in (50μM) were obtained from Calbiochem and used according to the supplier's recommendation. The PIP_3 Mass Strip kit was obtained from Echelon Biosciences.

Constructs. The dominant negative Akt construct K179M, T308A, S473A (Ramaswamy et al, 1999) was created by William Sellers, and obtained from Addgene (plasmid 9031). Flag tagged JAM-A and JAM-A mutant protein (DL1) overexpression constructs (described previously (Mandell et al, 2004)) were further altered using site directed mutagenesis. Silent mutations were designed to prevent shRNA suppression of exogenous JAM-A: 5' AG GTC GAG AGG AAA CTG CTC TG. For rescue experiments, endogenous level expression of JAM-A was achieved by transfection (with Lipofectamine 2000 according to manufacturers instruction) with 0.1μg Full length JAM-A or 1.0μg DL1 JAM-A per well (6 well dish). Cell counts were taken 72hrs post transfection.

Small Interfering RNA (siRNA). siRNA oligonucleotides were obtained from Dharmacon RNA Technologies (Lafayette, CO) including target 1, as described previously (Severson et al, 2009), targets 2 and 3: Human F11R (L-005033-00) and (MQ-005053-01-0002). Experiments in Fig 2 were performed using target 1, all other

experiments utilized targets 2 and 3, unless otherwise indicated. JAM-A knockdown and rescue experiments (Fig S1D) utilized the following siRNA target: 5' CA GTC GAG AGG AAA CTG TTG TG. siRNA for ILK; SASI_Hs01_00222546 (Sigma), siRNA for Akt, SASI_Hs01_00105954 and SASI_Hs02_00332190 (Sigma). Controls siRNA was purchased from QIAGEN (AllStars Negative Control). Transfections were performed using HiPerFect (QIAGEN) according to the manufacturer's instructions. Transfections consisted of a total concentration of 50 nM siRNA and assays were performed 48 h post transfection.

TCF/Lef and β-catenin Reporter Expression TCF reporter construct activity was measured using Dual Luciferase Reporter system (Promega, Madison WI) according to the manufacturer's instructions. β-galactosidase expression in BAT-gal mice was visualized by 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) staining, as described previously (Byrne et al, 1994). Briefly, tissue specimens were fixed with 3.7% wt/vol paraformaldehyde, incubated with X-gal staining solution overnight, and then postfixed before further processing.

Statistics. Paired Student's t test or one-way ANOVA with Bonferroni's post test was used to analyze the data. Results are displayed as mean ± standard error of the means (SEM) or standard deviation as indicated. $p<0.05$ was the threshold for statistical significance. Post hoc analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

Supplemental Figure Legends

Fig S1. (A) Apoptotic marker caspase-3 (cleaved) staining in Wt and JAM-A-/- mice. (B) Western blot showing overexpression of full length JAM-A (FL), and dimerization-defective mutant JAM-A (DL1). (C) SKCO15 cell proliferation was assessed by BrdU incorporation with or without overexpression of full length JAM-A or JAM-A DL1. (SEM n=3, *p<0.1, FL vs control and DL1, by one way ANOVA, Bonferroni's post test). (D) Western blot showing siRNA mediated suppression of endogenous JAM-A (lower arrow) but not mutant full length JAM-A (siRNA immune JAM-A, upper arrow). Also, expression of mutant full length JAM-A was titrated to approximate endogenous levels. Base-pair substitutions introduced into FL JAM-A in order to make this construct siRNA immune are indicated in RED. (E) Cell counts of control or siRNA JAM-A treated SKCO15 cells, either with or without expression of siRNA-immune full length JAM-A (FL) or dimerization-defective mutant JAM-A (DL1) (SEM n=2, *p<0.05, ANOVA).

Fig S2 (A) Suppression of JAM-A increases nuclear localization of pβ-catenin 552. Subcellular separation of cytosolic and nuclear proteins as shown by western blot in siRNA or siJAM-A transfected SKCO15 cells. Tubulin and PML are included as cytosolic and nuclear markers respectively. (B) SKCO15 cells transfected with the TOPFLASH TCF reporter construct show activity similar to control conditions in the presence of Doxycycline (Dox, 4μg/ml).

Fig S3 (A) JAM-A suppression mediated increase in TCF signaling is attenuated by treatment with Akt inhibitor Akt-in. Inhibition of Akt activation following loss of JAM-A is shown on the right. (B) JAM-A-suppression induced TCF signaling is attenuated with Akt knockdown. SKCO15 cells transfected with the TOPFLASH TCF reporter and control siRNA or siJAM-A, siAkt, or siJAM-A/siAkt. (C) Quantitation of Akt p308 in lysates from colon tissue treated with vehicle or triciribine (Tri).

Fig S4 (A) Depletion of JAM-A increases the cytosolic pool of phosphatidylinositol (3,4,5)-triphosphate (PIP_3), as determined using a PIP_3 Mass Strip assay kit. (B) Depletion of PI-3 kinase p110 inhibits Akt activation and β-catenin transcriptional activity following loss of JAM-A. (C) Overexpression of dominant negative Akt (AktDN) completely blocks β-catenin/TCF transcriptional activation, independent of JAM-A.

Fig S5 (A) Depletion of JAM-A inhibits PTEN in vitro, as indicated by increased PTEN phosphorylation. (B) Expression of PTEN is decreased in mucosal lysates of JAM-A deficient mice in vivo. (C) and (D) Depletion of PTEN in SKCO15 cells increases β-catenin transcriptional activity and Akt activation. Inhibition of Akt using triciribine inhibits transcriptional activation of β-catenin following loss of PTEN.

Supplemental References

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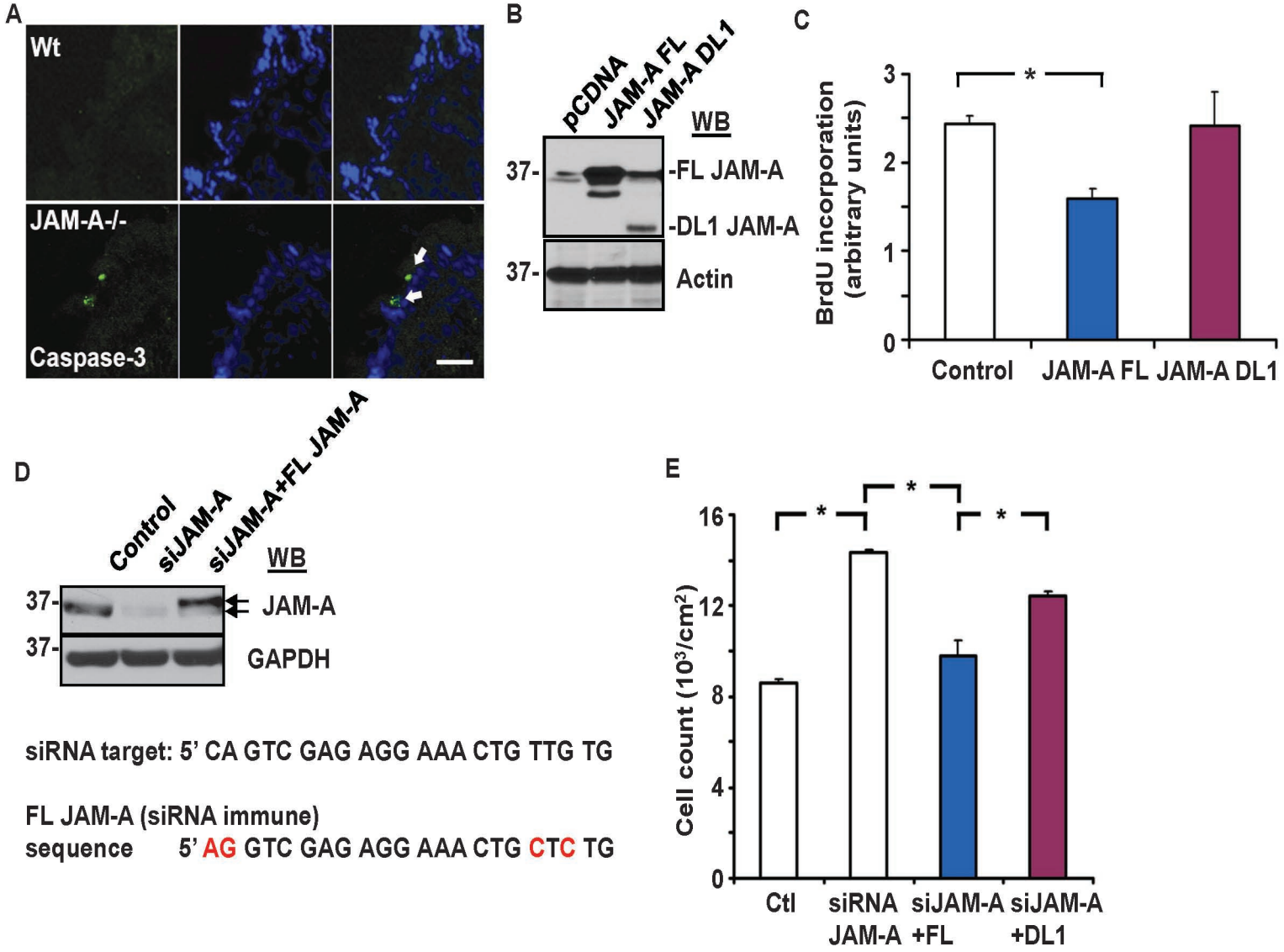
Supplemental Figure 1



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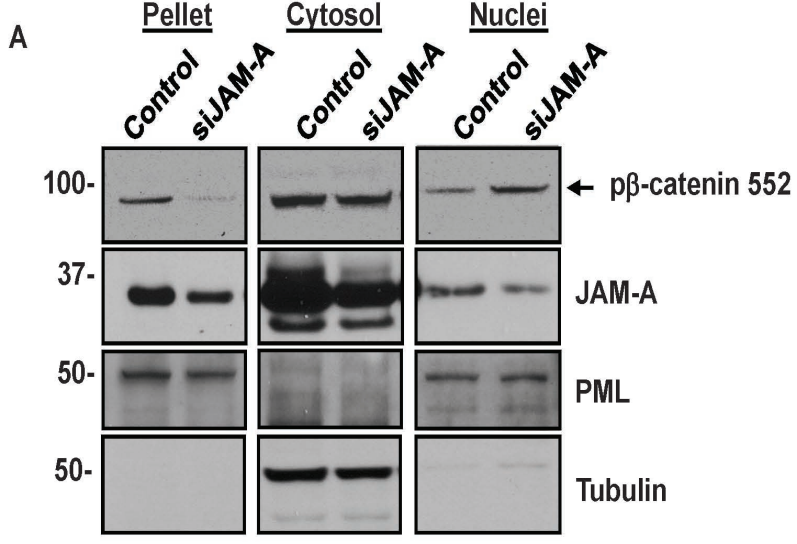

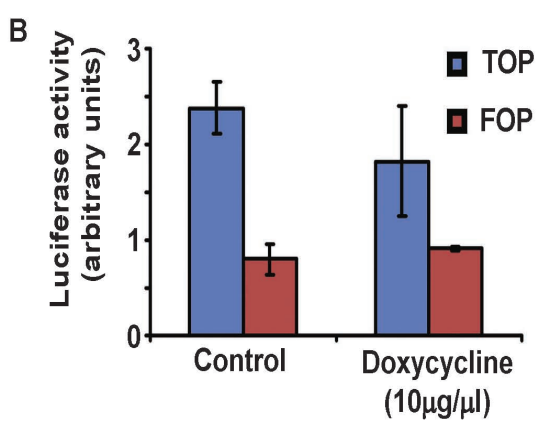

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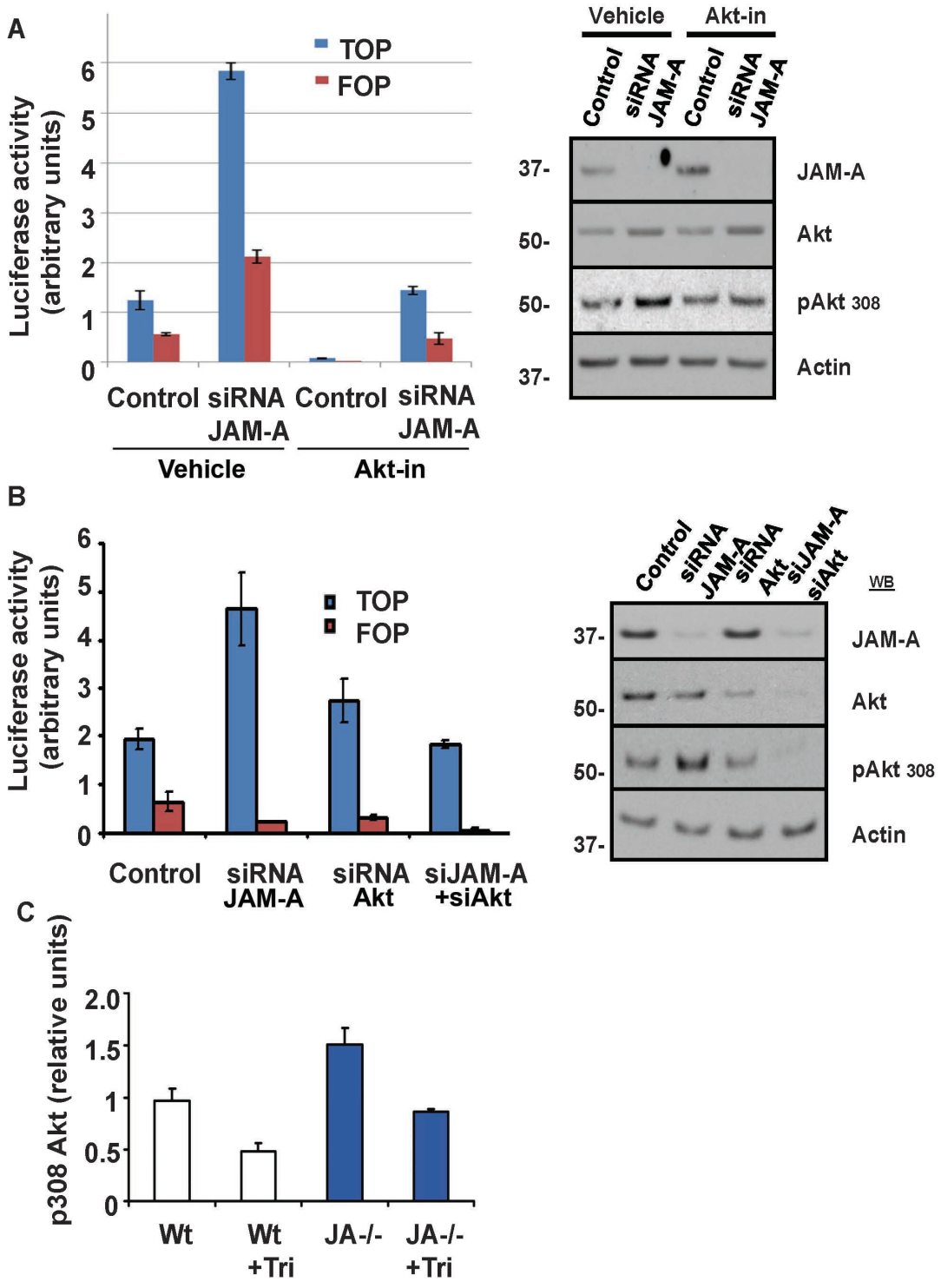

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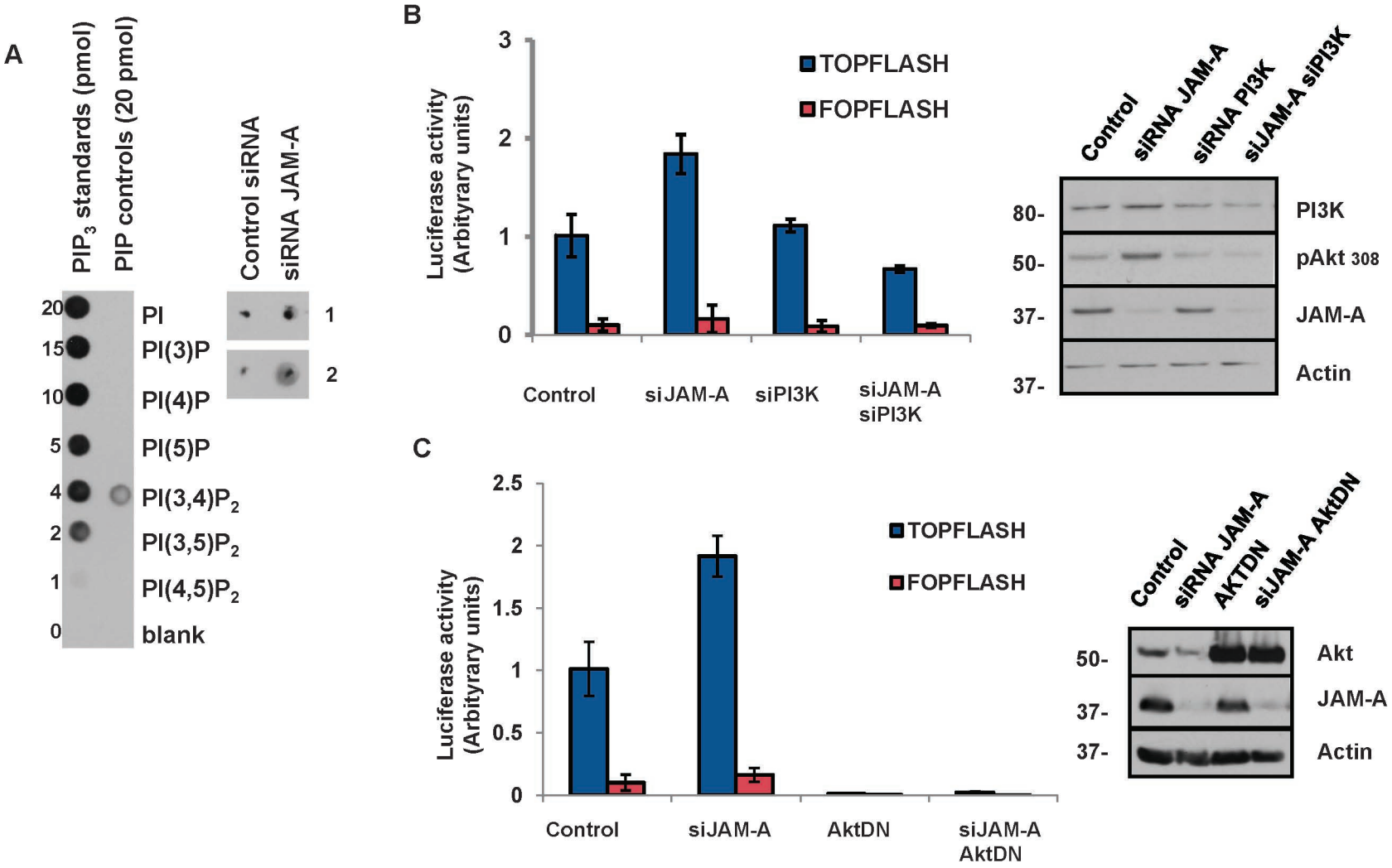

Supplemental figure 5