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Cancer Research

PIPKIγ Regulates β-Catenin Transcriptional Activity Downstream of Growth Factor Receptor Signaling

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Abstract

Increased β-catenin transcriptional activity downstream of the Wnt/Wingless signaling pathway has been observed in many human tumors, most notably colorectal carcinomas. However, β-catenin activation is also observed in many human malignancies with no observable Wnt activity. Wnt-independent pathways that activate β-catenin remain undefined, yet have the potential to play a significant role during tumorigenesis. Here, we report that phosphotidylinositol phosphate kinase Iγ (PIPKIγ), an enzyme that generates phosphoinositide messengers *in vivo*, directly associates with β-catenin and increases β-catenin activity downstream of growth factor stimulation. PIPKIγ expression and kinase activity enhance β-catenin phosphorylation on residues that promote nuclear importation and transcriptional activity. Lastly, we show that β-catenin is required for PIPKIγ-dependent increased cell proliferation. These results reveal a novel mechanism in which PIPKIγ expression and catalytic activity enhance β-catenin nuclear translocation and expression of its target genes to promote tumorigenic phenotypes. *Cancer Res; 71(4); 1282–91. ©2011 AACR.*

Introduction

β-Catenin is a potent oncogene with dual functions in the cell (reviewed in ref. 1). It was first identified as an essential component in mediating E-cadherin–based cell–cell contacts. Within the adherens junction, β-catenin links the cytoplasmic domain of E-cadherin to the actin cytoskeleton via its interaction with α-catenin. In addition, β-catenin acts as a transcriptional activator in many signaling pathways, including the Wnt/Wingless, epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) signaling pathways (2–5). In unstimulated cells, cytoplasmic levels of β-catenin are kept in check by a degradation complex consisting of axin, glycogen synthase kinase-3β (GSK-3β), and the adenomatous polyposis coli protein (APC; ref. 1). β-Catenin phosphorylation by GSK-3β results in binding β-TRCP/HOS and its ubiquitylation by $SCF^{\beta\text{-TRCP}}$, leading to proteasomic degradation (6). Wnt stimulation prevents GSK-3β–mediated phosphorylation and degradation and instead leads to unique phosphorylation events on β-catenin, resulting in its translocation to the nucleus where it interacts with different transcription factors, most notably members of the TCF/LEF family, to control the expression of its target genes, including *c-Jun, c-Myc*, and *cyclin D1* (2, 3, 7).

The role of β-catenin in promoting tumorigenesis has been well documented (8). Increased β-catenin activity can lead to uncontrolled cell proliferation, altered cell migration, and changes in cell polarity (8, 9). Activating mutations within the Wnt signaling pathway occur in more than 90% of colorectal cancers and have been observed in hepatocellular carcinomas (1). In addition, roles for β-catenin in promoting breast and prostate cancer are emerging (10, 11). However, activating mutations within the Wnt signaling pathway, including *CTNNB1* (which encodes β-catenin), is less frequent in many epithelial-derived cancers, including breast cancer (6, 12), although increased levels of nuclear β-catenin are still frequently observed (13). This indicates that other signaling pathways can induce β-catenin activation during tumorigenesis.

A hallmark of tumorigenesis is the acquisition of invasive capabilities (14). Emerging evidence supports an epithelial-to-mesenchymal transition (EMT) during tumor progression, which results in the loss of E-cadherin–based contacts and increased invasiveness (15, 16). Disruption of E-cadherin–mediated adhesions correlates with increased β-catenin–dependent transcription and poor prognosis in cancer patients (17). Forced expression of E-cadherin is able to reduce the tumorigenicity of epithelial-derived cancer cells (18), at least in part, due to its ability to sequester β-catenin in the cytoplasm. Stimulation of cells with growth factors, such as EGF or TGF-β, can induce the disruption of E-cadherin–based contacts and promote EMT (19, 20). Activation by these growth factors also increases the activity of many kinases, including Src, protein kinase B (PKB or AKT), and protein kinase A (PKA), all of which can increase β-catenin nuclear localization and activity (21–24).

Phosphoinositides are a group of signaling molecules that affect a vast array of cellular processes, including polarization,

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directional migration, gene expression, and proliferation (25, 26). Phosphotidylinositol phosphate kinase Iγ (PIPKIγ) phosphorylates phosphatidylinositol 4-phosphate to generate phosphatidylinositol 4,5-bisphosphate (herein referred to as PIP_2) *in vivo* (26). In addition to its use as a precursor to 3 separate and essential second messengers [inositol 1,4,5-trisphosphate (IP_3), 1,2-diacylglycerol (DAG), and PIP_3], PIP_2 directly binds many protein effectors, regulating their activity. PIPKIγ can alternatively splice to give rise to at least five different protein isoforms, known as PIPKIγi1–5 (27). While the localization of endogenous isoforms is distinct, overexpressed PIPKIγs lose their localization specificity and can be found ubiquitously throughout the cell (27). However, understanding the effects of overexpression is of critical importance, as recently it was shown that PIPKIγ overexpression in breast cancer patients correlated with decreased survival (28) and increased PIPKI activity was observed in hepatocellular carcinomas (29).

PIPKIγ expression correlates with poor survival in breast cancer patients. In defining a cellular mechanism for this clinical observation, we found that PIPKIs associate with β-catenin and that increased PIPKIγ-dependent cellular proliferation requires β-catenin. Phosphoinositide messengers derived from PIPKIγ expression hyperactivate the β-catenin transcriptional machinery. In addition, PIPKIγi2-generated phosphoinositide messengers result in the hyperphosphorylation and increased nuclear accumulation of β-catenin. Finally, we show that PIPKIγi2 expression enhances the activation of β-catenin downstream of growth factor stimulation.

Materials and Methods

Please see Supplemental Experimental Procedures for the following sections: "Reagents"; "Cell culture and transfection"; "Lysate preparation and immunoblotting"; "Immunofluorescence and microscopy"; and "GST affinity pull-down assays."

Luciferase reporter assays

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. HeLa Tet-Off cells stably expressing PIPKIγ constructs have been described elsewhere (30). All other cell lines were obtained from American Type Culture Collection and maintained in passage for less than 3 months. To examine the effects of PIPKIs on β-catenin transcriptional activity, cells were transfected with Super8-XTOPFLASH (Millipore), pRL-null Renilla (Promega), and pCMV-HA alone or pCMV-HA containing WT or mutant PIPKI constructs. Eighteen to 24 hours after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and the Pharmingen Monolight 3010 Luminometer (BD Pharmingen). Reporter expression was normalized to cotransfected Renilla luciferase activity. To determine whether E-cadherin could inhibit the activation of β-catenin, pCMV-myc, or pCMV-myc E-cadherin cytoplasmic domain was included at the time of transfection. For growth factor receptor activation experiments, cells were transfected as stated. Cells were serum starved in DMEM + 0.5% FBS for 12 to 24 hours and stimulated with the appropriate growth factor. Twenty-four hours later, β-catenin activity was assayed as stated.

Cell growth assays

Oligonucleotide sequences used for generation of *PIPKIγ* short hairpin RNA were GCCACCTTCTTTCGAAGAA (PIPKIγshRNA) and GCCTTCTTCGCTAAACGAA (ConshRNA). Generation of replication-defective infectious viral particles and the transduction of the cells were carried out following the protocol provided by Addgene, as described previously (31, 32). In brief, synthesized oligonucleotides were annealed and cloned into *Hpa*I and *Xho*I sites of pLL3.7 vector (Addgene). Stabl3 competent cells (Life Technologies) were used for transformation and DNA purification. For generation of viral particles, lentiviral vector with accessory plasmids (pCMV-VSVG, pRSV-Rev, and pMD2.G) were cotransfected into HEK 293T cells, using calcium phosphate. Conditioned medium was collected 48 hours posttransfection, filtered through a 0.45-μmol filter, and viral particles were concentrated by centrifugation at 24,000 rpm in Beckman SW28 centrifuge (Beckman Coulter) for 2 hours at 4°C. Subconfluent HeLa cells were infected with viral supernatant in the presence of 0.5 μg/mL polybrene (Sigma). Infected cells were sorted using a cell sorter (GFP expression driven by CMV) or individual clones were isolated. PIPKIγ knockdown was examined by immunoblotting. To monitor proliferation, infected cells were seeded into 12-well culture plates at a density of 1,000 cells/well. Manual cell counting was done every 24 hours for 8 days. Cell numbers were counted from at least 3 wells for each cell type and expressed as mean ± SD from one representative experiment.

To monitor cell proliferation in HeLa Tet-Off cells in conjunction with β-catenin knockdown, HeLa Tet-Off cells expressing empty vector (EV) or PIPKIγi2 were grown in DMEM + 10% FBS without doxycycline to induce PIPKIγi2 expression. An equal number of cells were transfected with β-catenin–validated or control stealth siRNA oligos (Invitrogen), using Oligofectamine (Invitrogen), according to the manufacturer's instructions, and proliferation was monitored for 48 hours posttransfection. The extent of β-catenin knockdown was monitored at the 48-hour time point. Manual cell countings from at least 3 wells for each cell type and time point were expressed as mean ± SD.

Results

PIPKIγ associates with β-catenin independent of E-cadherin

PIPKIγ associates with a region on E-cadherin that lies within the previously defined β-catenin–binding region (30). Because of this, an interaction between PIPKIγ and β-catenin was investigated. β-Catenin coimmunoprecipitated with transiently transfected HA-tagged PIPKIα, PIPKIγi1, PIPKIγi2, and $PIPKI\gamma i2^{KD}$ from HeLa cells but not from cells transfected with EV (Fig. 1A). Because both β-catenin and PIPKIγ splice variants associate with E-cadherin and E-cadherin is expressed at low levels in these cells (data not shown), we determined whether E-cadherin was required to generate an association between β-catenin and PIPKIs. Endogenous PIPKIγ and β-catenin

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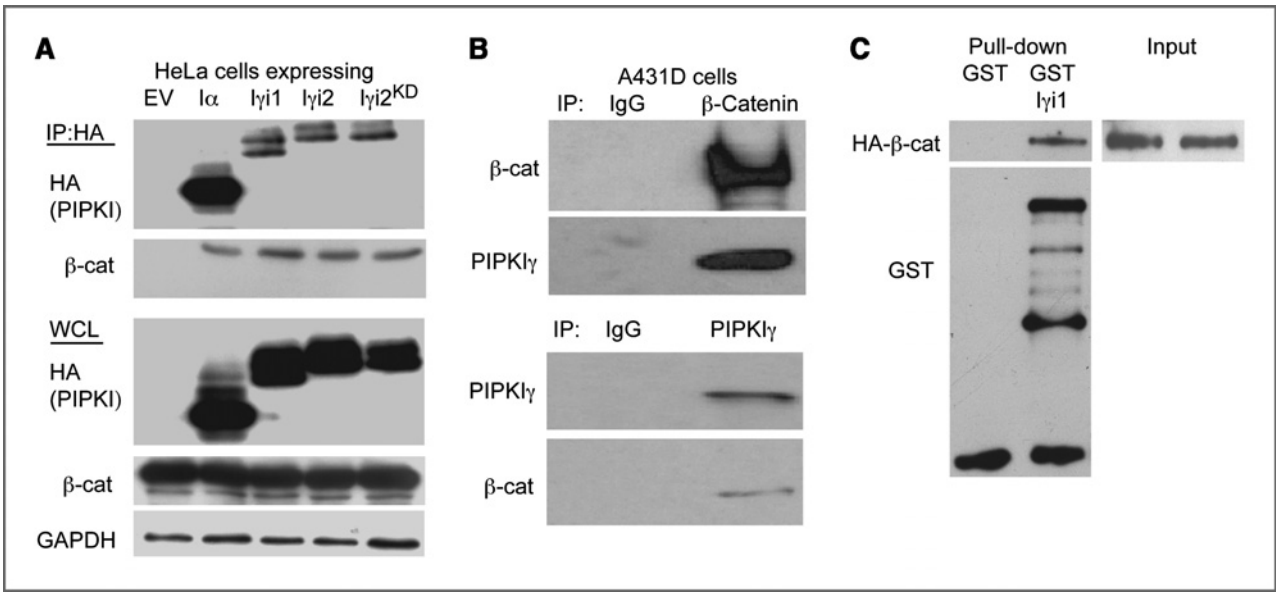

Figure 1. PIPKIγ directly associates with β-catenin independent of E-cadherin. A, HeLa cells were transiently transfected with EV or the HA-PIPKI shown and an interaction with β-catenin was assayed by immunoprecipitation (IP), using anti-HA antibody. Whole cell lysates (WCL) were probed with anti-HA, anti-β-catenin, and anti-GAPDH to monitor transfection and protein levels. B, endogenous β-catenin and PIPKIγi1 were coimmunoprecipitated from A431D cells. C, recombinant GST or GST-β-catenin was assayed for interaction with purified PIPKIγi1 by *in vitro* pull-down assays. A–C, representative blots of at least 3 separate experiments. β-cat, β-catenin.

coimmunoprecipitated from A431D and CHO-K1 cells, which lack classical cadherins (33, 34), indicating that this interaction is independent of cadherins *in vivo* (Fig. 1B; data not shown). Finally, to test whether or not β-catenin and PIPKIγ were able to directly associate, GST pull-down assays using recombinant proteins were used, which showed that β-catenin and PIPKIγi1 are able to directly interact (Fig. 1C). The PIPKIγi1 protein does not contain a C-terminal extension and its sequence is conserved in all PIPKIγ splice variants (27). The combined data show that PIPKIγ interacts directly and separately with both β-catenin and E-cadherin and that the β-catenin–interacting region on PIPKIγ lies within the conserved region. As PIP kinases often regulate the proteins they associate with, the impact of PIPKIs on β-catenin activity was investigated.

PIPKIγ stimulates β-catenin transcriptional activity

To examine the effect of PIPKIγ on the transcriptional activity of β-catenin, TOPflash luciferase reporter constructs (35) were transfected into HeLa cells expressing PIPKIs, and transcriptional activity was quantified (22). When grown in the presence of serum, PIPKIα and each PIPKIγ isoform (PIPKIγi1–5) stimulated β-catenin transcriptional activity in HeLa cells (Fig. 2A). Interestingly, this increased activity required the generation of phosphoinositides, as kinase-inactive ("kinase-dead," KD) mutants of PIPKIs, PIPKIα^{KD}, PIPKIγi2^{KD}, or PIPKIγi4^{KD} failed to stimulate β-catenin transcriptional activity. Furthermore, PIPKIγ^{KD} isoforms diminished β-catenin activity, indicating that they act in a dominant-negative fashion (Fig. 2A). Expression of PIPKIγ isoforms with tetracycline-inducible cell lines (HeLa Tet-Off) showed that PIPKIγ stimulated β-catenin activity to the same or greater extent as transient transfection experiments and PIPKIγ^{KD} mutants diminished β-catenin activity to that in uninduced cells (Supplementary Fig. S1A). Transient transfection of PIPKIs in HEK293T cells also stimulated β-catenin transcriptional activity, indicating that this signaling event was not exclusive to HeLa cells (Supplementary Fig. S1B).

PIPKIγ can modulate the adherens junction and E-cadherin trafficking in epithelial cells (30). In addition, E-cadherin expression can inhibit β-catenin activity in mesenchymal-like cells (16). We determined whether PIPKI expression could stimulate β-catenin transcriptional activity in polarized epithelial cells. Surprisingly, expression of PIPKIα or PIPKIγ isoforms in MDCK cells (Madin Darby canine kidney cells) did not significantly increase β-catenin transcriptional activity (Fig. 2B). We then tested whether reexpression of E-cadherin could block β-catenin activity in HeLa cells grown in the presence of serum. The E-cadherin cytoplasmic tail domain (ECD-CT; ref. 30) was expressed because full-length E-cadherin protein is quickly internalized and degraded in HeLa cells (data not shown), thus eliminating the necessity for oligomer homophilic interactions between adjacent cells for E-cadherin complex assembly. ECD-CT expression in HeLa cells blocked PIPKI activation of β-catenin (Fig. 2C). Furthermore, ECD-CT expression in cells overexpressing PIPKIγi2 significantly reduced the association between PIPKIγi2 and β-catenin (Fig. 2D).

PIPKIγ expression and catalytic activity enhance β-catenin nuclear accumulation

Wnt or growth factor stimulation can lead to the accumulation of β-catenin in the nucleus, where it regulates gene expression (2, 3, 9). β-Catenin localization changes were observed upon expression of PIPKIγ. The activation of β-catenin

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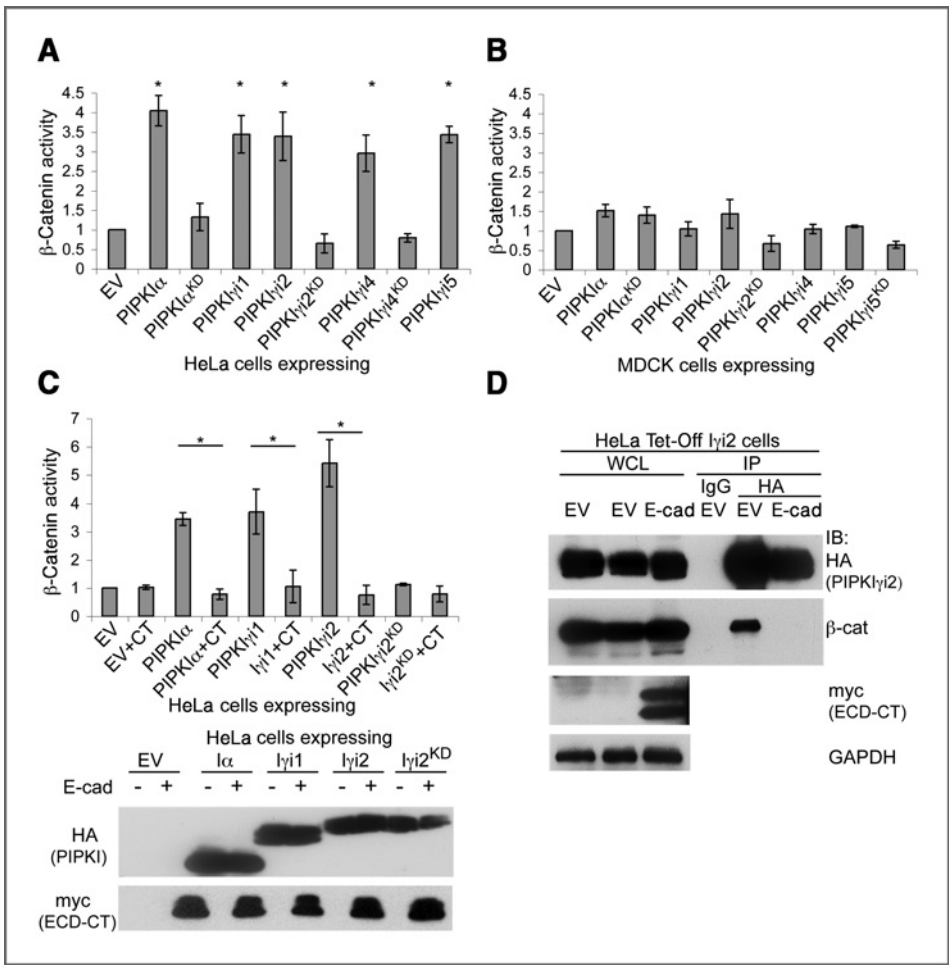

Figure 2. PIPKIγ expression stimulates β-catenin transcriptional activity in mesenchymal-like cells lacking E-cadherin. β-Catenin transcriptional activity was measured in (A) HeLa or (B) MDCK cells transiently transfected with either EV or the HA-PIPKI shown. *, $P \leq 0.001$. C, HeLa cells were treated as in A; however, either EV or E-cadherin cytoplasmic domain (CT) was included in the transfection where indicated. *, $P \leq 0.01$. Representative Western blots show the expression of PIPKIs with myc-ECD-CT. D, HeLa Tet-Off cells expressing HA-PIPKIγi2 were transfected with EV or myc-ECD-CT where indicated. Lysates of equal protein concentration were immunoprecipitated using anti-HA to determine how the PIPKIγi2/β-catenin association was affected upon expression of E-cadherin. Shown is a representative blot of 4 separate experiments. For all graphs, $n \geq 3$; error bars, SD. β-cat, β-catenin; E-cad, E-cadherin.

by PIPKIγi1 and PIPKIγi2 was the focus, but we do not discount the possibility that PIPKIα, PIPKIγi4, or PIPKIγi5 can also result in similar cellular phenotypes. Wild-type or PIPKIγ mutants were expressed in HeLa cells, and immunofluorescence was used to visualize changes in the localization of endogenous levels of β-catenin. Transient expression of PIPKIγi2 increased nuclear accumulation of β-catenin more than 4-fold when compared with cells transfected with EV (Fig. 3A–C). This accumulation required catalytic activity as expression of PIPKIγi2^{KD} failed to increase nuclear β-catenin levels (Fig. 3). Similar results were observed in HeLa Tet-Off cells stably expressing PIPKIγi2 or PIPKIγi2^{KD} (Supplementary Fig. S2A and B). In addition, both transient and stable expression of PIPKIγi1 or PIPKIγi1^{KD} in HeLa cells revealed a similar phenotype (data not shown), indicating that PIPKIγ overexpression stimulated the nuclear accumulation of β-catenin in cells grown in the presence of serum.

PIPKIγ stimulates the phosphorylation of β-catenin at sites known to initiate nuclear translocation and transcriptional activation

Accumulation of cytoplasmic levels of β-catenin and its nuclear importation and transcriptional activation are all enhanced by phosphorylation (21). Using HeLa Tet-Off cells, we found that PIPKIγi2 expression increased β-catenin phosphorylation on Ser552 and Ser675 (Fig. 4). Furthermore, phosphorylation levels at these sites decreased upon expression of PIPKIγi2^{KD} (Fig. 4). Surprisingly, neither PIPKIγi2 nor PIPKIγi2^{KD} expression significantly altered β-catenin protein levels, nor did their expression alter β-catenin phosphorylation on Ser33, Ser37, and Ser45, sites known to be involved in the β-catenin proteasomal degradation pathway (ref. 36; Fig. 4). β-Catenin activation results in transcriptional changes in target genes. Consistent with this, upon expression of PIPKIγi2, an increase in the protein levels of c-jun, fra-1, TCF-1/LEF1, and cyclin D1, known targets of β-catenin transcriptional activity, was observed (refs. 7, 37, 38; Fig. 4). However, PIPKIγi2 expression in HeLa cells did not result in increased levels of Oct-4 or vimentin (data not shown).

PIPKIγ enhances β-catenin activity upon growth factor stimulation

Overexpression of PIPKIγ correlates with increased metastasis and poor prognosis in breast cancer patients, and this correlated with the expression of the EGF receptor HER1 and HER2 (28). In addition, many growth factors, such as EGF and

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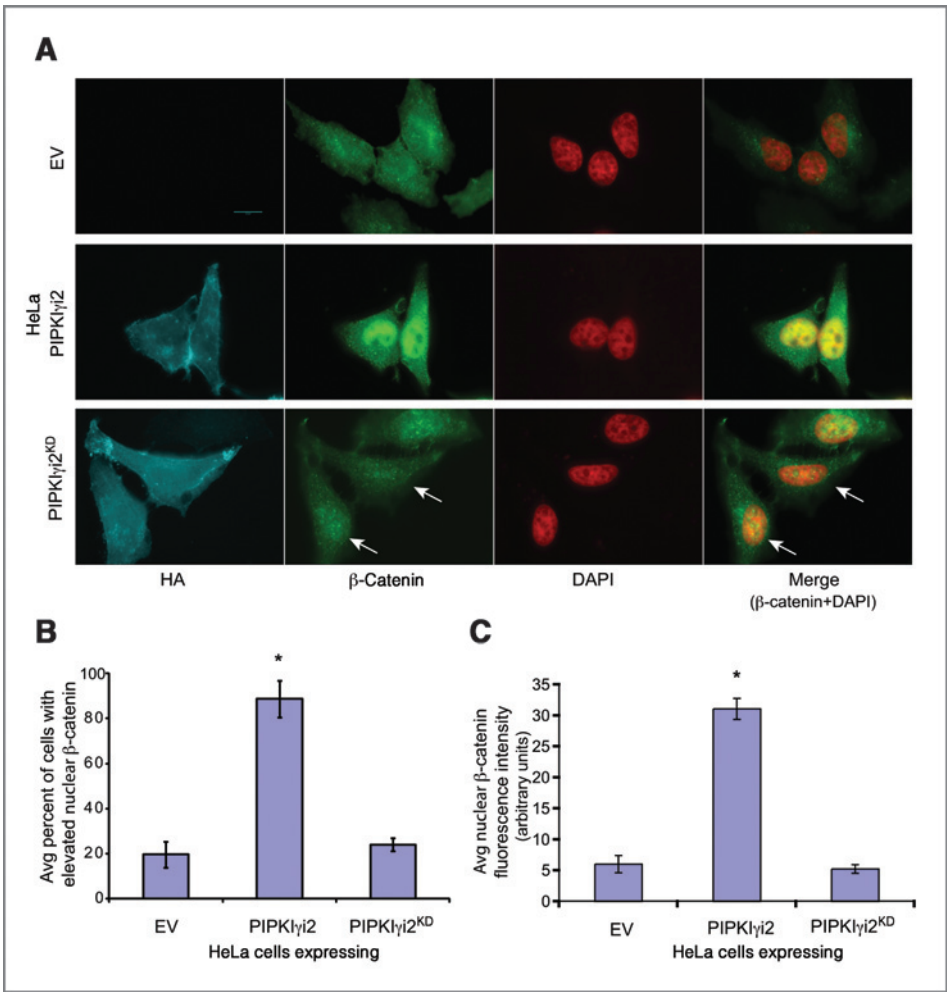

Figure 3. PIPKIγi2 expression and activity increase β-catenin nuclear accumulation. A, HeLa cells grown on glass coverslips for 24 hours were transiently transfected with EV or HA-tagged PIPKIγi2 or PIPKIγi2^{KD} where indicated. About 24 hours after transfection, cells were fixed and stained with anti-HA to monitor PIPKIγ-expressing cells, anti-β-catenin, and DAPI (4',6-diamidino-2-phenylindole; pseudocolored red). All images were taken with a 60× objective. Arrows point to PIPKIγi2^{KD}-expressing cells. B, graphical representation of the effects of PIPKIγi2 expression on nuclear β-catenin levels. Shown is the average ($n = 3$) percentage of cells counted that showed increased nuclear β-catenin staining. C, shown is a graphical representation of the average ($n = 3$) nuclear β-catenin fluorescence. *, $P \leq 0.05$; error bars, SD.

TGF-β, stimulate β-catenin transcriptional activity (39, 40). We set out to determine whether PIPKIγ played a role in the activation of β-catenin downstream of growth factor receptor activation. For this, β-catenin transcriptional activity was quantified in HeLa cells transiently expressing wild-type or kinase inactive mutants of PIPKIα or PIPKIγi2. After transfection of the PIPKI, TOPflash, and Renilla reporter constructs, cells were serum starved and then stimulated with EGF, HGF, or TGF-β1/2. Increased PIPKIγi2 expression greatly enhanced β-catenin–dependent transcriptional activity following treatment with growth factors (Fig. 5A). This increase in β-catenin activity was dependent on its catalytic activity, as PIPKIγi2^{KD} failed to stimulate β-catenin (Fig. 5A). Surprisingly, the activation of β-catenin following stimulation with EGF, HGF, or TGF-β was PIPKIγ specific, as PIPKIα expression did not enhance β-catenin activity (Fig. 5A). EGF-mediated activation of β-catenin was also enhanced in HeLa Tet-Off cells stably expressing PIPKIγi2 and severely diminished in cells expressing PIPKIγi2^{KD} (Supplementary Fig. S3C). Stimulation of HeLa cells with EGF, HGF, or TGF-β resulted in expected activation of signaling molecules within these pathways, as evidenced by Western blotting using activation-specific antibodies (Supplementary Fig. S3B). These results indicate that PIPKIγ enhances β-catenin transcriptional activation downstream of growth factor receptor stimulation.

The classical Wnt pathway leads to the activation of β-catenin. Increased expression of PIPKIγi2 or PIPKIγi2^{KD} did not significantly affect β-catenin activity following Wnt stimulation (Supplementary Fig. S3A). However, Wnt-dependent activation of β-catenin was moderately but consistently enhanced upon PIPKIα expression and decreased upon PIPKIα^{KD} expression, though the regulation was not statistically significant (Supplementary Fig. S3A). Recently, PI4KIIα and PIP5KIβ were shown to be required for LRP6 phosphorylation downstream of Wnt stimulation (41). PIPKIα expression enhanced β-catenin activity when cells were grown in sera (Fig. 2A). This suggests that the PIPKIα modulates β-catenin within some agonist-, but not growth factor-, stimulated pathways.

Next, we determined whether growth factor stimulation induced β-catenin phosphorylation patterns similar to PIPKIγi2 expression. EGF stimulation increased EGFR phosphorylation on Tyr1173 and Tyr1086 (Fig. 5B and Supplementary Fig. S3B). In addition, EGF stimulation increased

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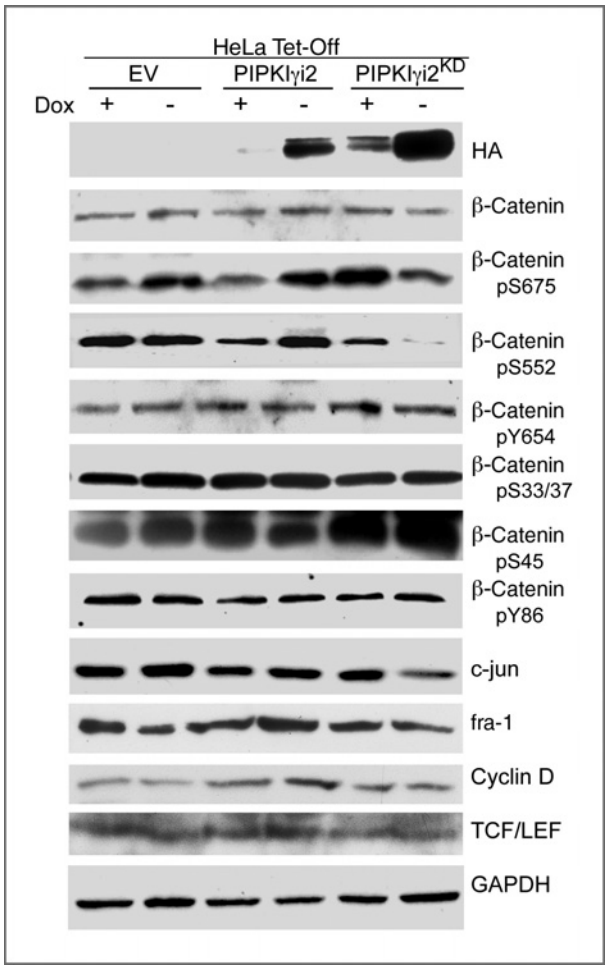

Figure 4. PIPKIγ expression stimulates β-catenin phosphorylation at sites known to initiate nuclear translocation and transcriptional activation. HeLa Tet-Off cells stably expressing EV or HA-tagged PIPKIγi2 or PIPKIγi2^{KD} were maintained in DMEM + 10% FBS + doxycycline. To initiate PIPKIγi2 expression, media was replaced with fresh media ± doxycycline where indicated. About 24 hours later, whole cell lysates (WCL) were prepared and probed with the antibodies mentioned. Shown are representative blots of 3 experiments. Dox, doxycycline.

β-catenin phosphorylation on Ser552 and Ser675 (Fig. 5B). Surprisingly, the stimulation of HeLa cells with EGF resulted in a decreased association between endogenous PIPKIγ and β-catenin, as evidenced by coimmunoprecipitation experiments using pan-PIPKIγ antibodies (Fig. 5C). In cells overexpressing PIPKIγi2, EGF treatment enhanced EGFR phosphorylation and β-catenin phosphorylation on Ser552 and Ser675 (Fig. 5D). Furthermore, EGF treatment increased the association between overexpressed PIPKIγi2 and β-catenin (Fig. 5E).

β-Catenin is required for tumorigenic phenotypes associated with PIPKIγ expression

Previously, PIPKIγ expression in breast cancers was shown to inversely correlate with patient survival (28). The cellular mechanisms by which PIPKIγ activates β-catenin suggest that these molecules could cooperate to promote tumorigenesis. To further explore this possibility, the growth rate of cells expressing PIPKIγ was monitored. Knockdown of PIPKIγ using siRNA in MDA-MB-231 breast cancer cells results in a significant decrease in proliferation (28). Furthermore, PIPKIγ expression in these cells enhanced β-catenin activity, a result dependent on its catalytic activity (Fig. 6A). In addition, lentiviral-mediated knockdown of PIPKIγ in HeLa cells also decreased proliferation (Fig. 6B). siRNA was used to knock down β-catenin in HeLa Tet-Off cells expressing EV or PIPKIγi2. When grown in the presence of serum, PIPKIγi2 expression significantly increased cell proliferation (Fig. 6C, Iγi2 control vs. EV control). This increase diminished upon treatment with β-catenin–specific siRNA (Fig. 6C). Surprisingly, knockdown of β-catenin in HeLa Tet-Off cells expressing EV had little effect on the growth rate when grown in the presence of serum (Fig. 6C). This shows that PIPKIγ expression enhances cell proliferation in epithelial-derived tumorigenic cells through its ability to activate β-catenin.

Discussion

The findings reported here represent a previously unrecognized role for PIPKIγ in regulating β-catenin transcriptional activity, providing a potential mechanism for understanding how PIPKIγ promotes tumorigenesis. Emerging evidence is defining significant roles for signaling pathways that activate β-catenin independent of Wnt and GSK-3β. Understanding how these pathways modulate β-catenin transcriptional activity is extremely important, as β-catenin can influence multiple aspects of tumorigenesis, including proliferation, migration, loss of cell polarity, and the establishment of a less differentiated state. Our findings mimic those of other groups in that the increased trascriptional activity of β-catenin is not accompanied by significant changes to its overall protein levels (3, 5, 22, 40, 42). β-Catenin activation via unique signaling pathways provides novel therapeutic targets for the treatment of malignancies.

Previously, PIPKIγ splice variants have been shown to interact directly with E-cadherin, modulating its trafficking to and from the plasma membrane (30). In normal epithelial cells, β-catenin is incorporated into cell–cell adhesions, where it provides a physical link between the cytoplasmic domain of E-cadherin and the actin cytoskeleton. Indeed, β-catenin is required for the establishment of cell polarity in normal epithelia (43). Upon depletion of Ca^{2+} or stimulation with growth factors, such as EGF, HGF, or TGF-β, E-cadherin is rapidly internalized, increasing cytoplasmic levels of β-catenin (21, 44). PIPKIγ could activate β-catenin by enhancing the endocytosis and degradation of E-cadherin, thereby preventing E-cadherin from sequestering β-catenin at the cell membrane. We observe that HeLa cells express low levels of E-cadherin that localizes to the plasma membrane at sites of cell–cell contact when cells are confluent. Expression of PIPKIγ in these cells could lead to higher levels of cytoplasmic β-catenin, thus allowing for a greater activation. Consistent with this model, overexpression of the E-cadherin C-terminal domain blocked PIPKIγ-mediated increase in β-catenin transcriptional activity. However, expression of PIPKIγ in normal

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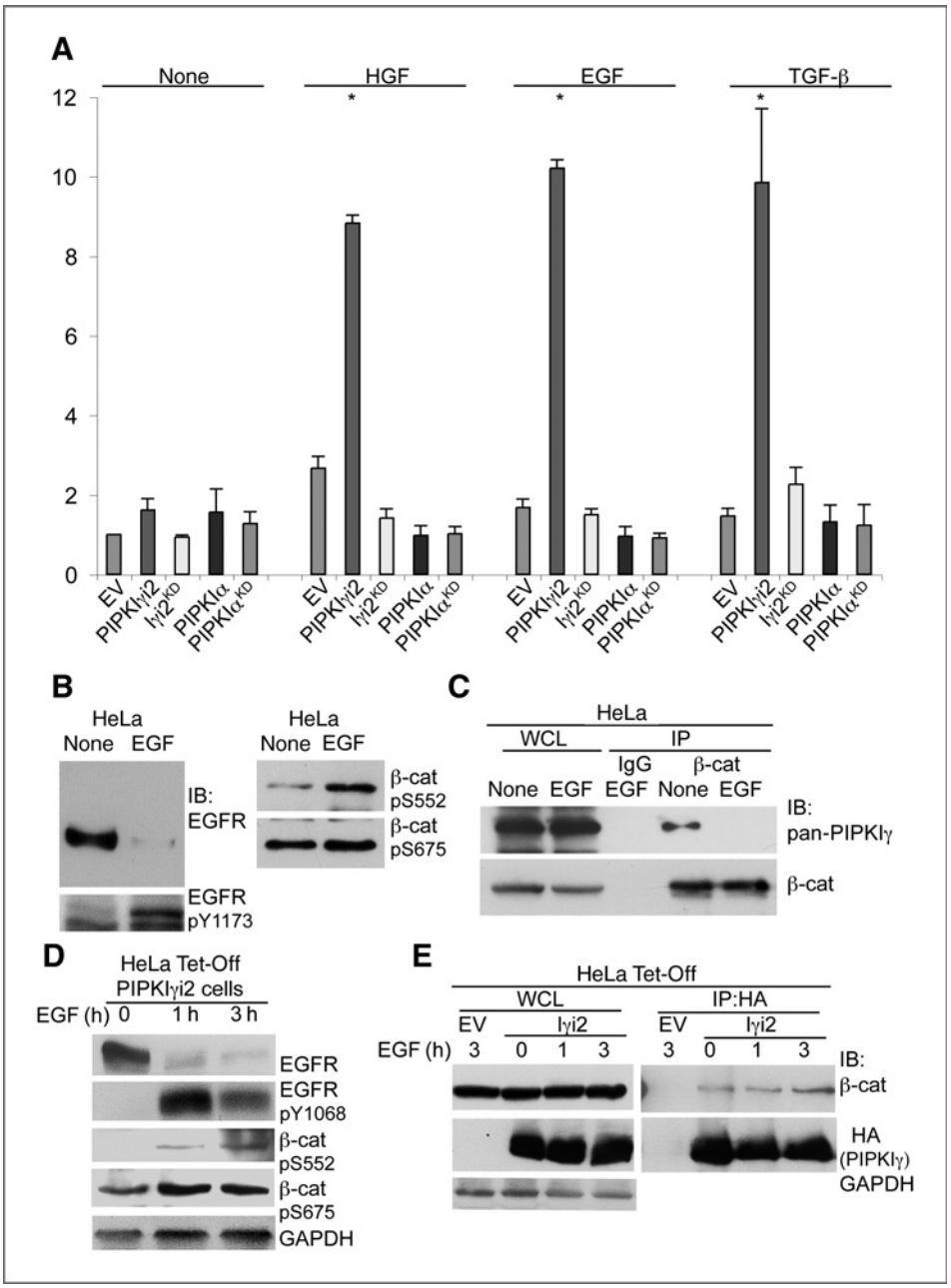

Figure 5. PIPKIγ activates β-catenin upon growth factor stimulation. A, β-catenin transcriptional activity was measured in HeLa cells transiently transfected with EV or the HA-PIPKI shown. About 24 hours after transfection, cells were serum starved and then left untreated (none) or stimulated with 1 nmol EGF, 50 ng/mL HGF, or 2 ng/mL TGF-β for 24 hours where indicated followed by cell lysis to measure β-catenin activity. *, $P \leq 0.01$; $n \geq 3$; error bars, SD. B, HeLa cells were serum starved and then left untreated (none) or stimulated with 1 nmol EGF for 2 hours where indicated. Whole cell lysates (WCL) were probed with the antibodies shown. C, cells were treated as in B. Following cell lysis, equal protein concentrations from none and EGF-treated WCLs were incubated with anti-β-catenin antibody. Immunoprecipitated β-catenin and coimmunoprecipitated PIPKIγ were detected with specific antibodies. D, HeLa Tet-Off PIPKIγi2 cells were maintained in medium without doxycycline to induce PIPKIγi2 expression. Cells were serum starved and stimulated with 1 nmol EGF for 0, 1, or 3 hours where indicated. WCLs were prepared and probed with the antibodies shown. E, cells were treated, as in D. Following cell lysis, WCLs of equal protein concentration were incubated with anti-HA antibody. Immunoprecipitated HA-PIPKIγi2 and coimmunoprecipitated β-catenin were detected with specific antibodies. HeLa Tet-Off cells transfected with the EV were used as a control during the IP. B–E, shown are representative blots of at least 3 experiments. β-cat, β-catenin.

epithelial cells, such as MDCKs, does not prevent E-cadherin translocation to the membrane or the formation of cell–cell contacts (30), nor does it significantly increase β-catenin activity, suggesting that other mechanisms are required to initiate E-cadherin endocytosis and/or β-catenin activation. In support of this, E-cadherin endocytosis is not sufficient to activate β-catenin when the β-catenin degradation complex is present (45).

Activation of some growth factor–stimulated signaling pathways has been shown to inhibit β-catenin degradation and/or enhance β-catenin stabilization and nuclear translocation (21). Because these pathways are often hyperactivated in cancers, this could explain why we see increased β-catenin activity upon PIPKIγ expression only in transformed cells. Therefore, under circumstances such as increased growth factor signaling or exposure to different extracellular stimuli, higher levels of the PIPKIγ protein results in a more enhanced activation of β-catenin. A recent report showed that β-catenin nuclear translocation occurs primarily in the peripheral cells of a tumor mass and correlates with an increased propensity for migration (46). β-Catenin transcriptional activity can enhance EMT, a process resulting in increased cellular migration and invasion associated with metastatic formation (47). We observe an enhanced activation of β-catenin in cells

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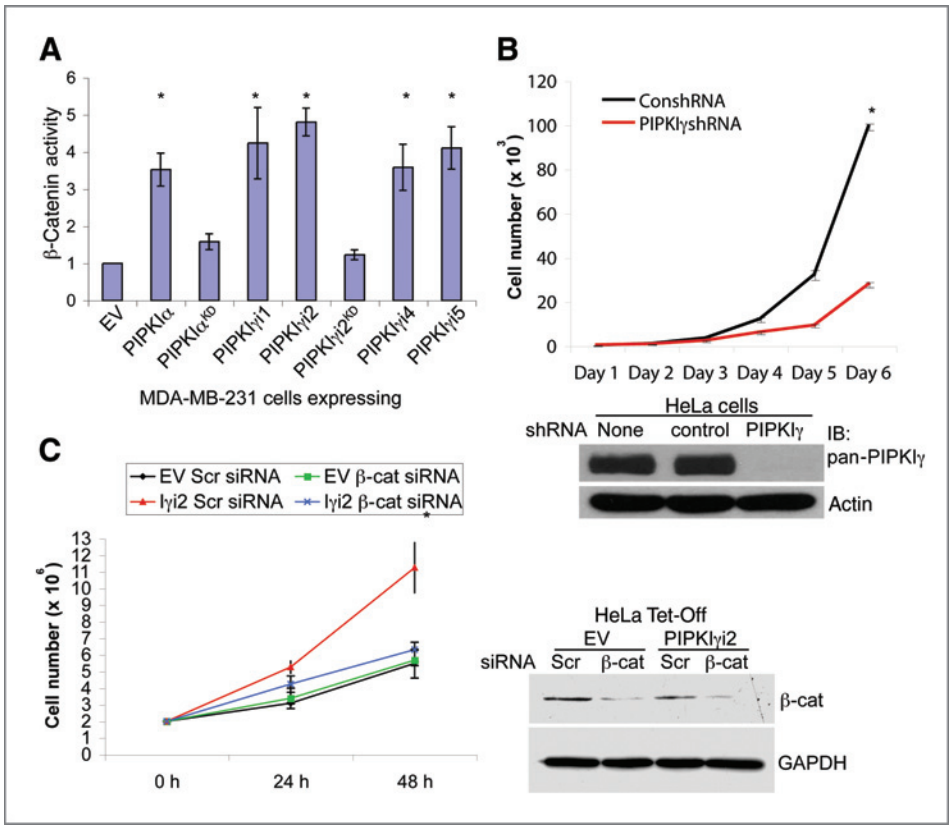

Figure 6. β-Catenin is required for tumorigenic phenotypes associated with PIPKIγ expression. A, β-catenin transcriptional activity was measured in MDA-MB-231 cells transiently transfected with TOPflash, pRL-null Renilla, and EV or the HA-PIPKI shown. Cells were grown for 24 hours in the presence of serum prior to lysis and quantification of luciferase activity. *, $P \leq 0.01$; $n \geq 3$; error bars, SD. B, HeLa cells were infected with lentivirus containing scrambled or PIPKIγ-specific shRNA and cell proliferation monitored for 6 days postinfection. Western blot analysis revealed the efficiency of PIPKIγ knockdown 72 hours postinfection. *, $P \leq 0.05$; $n \geq 3$; error bars, SD. C, HeLa Tet-Off parental and PIPKIγi2-expressing cells were treated with control or β-catenin–specific siRNA and cell proliferation monitored for 48 hours posttransfection. Shown are graphical representations of average cell growth at each time point. *, $P \leq 0.01$; $n = 3$; error bars, SD. Western blot analysis revealed knockdown efficiency. β-cat, β-catenin; Scr, scrambled.

overexpressing PIPKIγ upon treatment with TGF-β, a known inducer of EMT (20); however, we did not observe changes in the protein levels of EMT-associated genes *vimentin* and *Oct-4* in HeLa cells. A more thorough investigation of the global gene expression profiles of PIPKIγ-overexpressing cells will be required, although cell type–specific profiles will probably be found. It is likely that PIPKIγ can increase the activation of β-catenin on more than one level, as it could lead to its disassociation from E-cadherin and act within signaling pathways to increase its nuclear localization and transcriptional activity.

PIPKIγ catalytic activity is required to enhance β-catenin activation. Although it remains to be shown, this is presumably through its ability to generate phosphoinositide messengers, likely $PI4,5P_2$, as the other messenger known to be generated by PIPKIs *in vivo*, $PI3,4,5P_3$, utilizes a substrate that is present at miniscule cellular concentrations (26, 48). A spatial and temporal increase in $PI4,5P_2$ could enhance signaling pathways which utilize this messenger and also activate β-catenin. For example, PI3K could utilize pools of PIP_2 to generate higher amounts of PIP_3, thereby increasing or prolonging the activation of this signal. We observe an increase in the phosphorylation of β-catenin at Ser552 and Ser675 upon PIPKIγ expression. Ser552 can be phosphorylated by AKT (22), resulting in β-catenin accumulation in the nucleus and increased transcriptional activity. In addition, AKT is activated downstream of PIP_3 generation by PI3K, whose activation within cells following stimulation by growth factors, such as EGF, HGF, and TGF-β, has been well documented (49).

Phosphorylation of β-catenin at Ser552 and Ser675 is also mediated by cyclic AMP–dependent PKA, enhancing β-catenin activity (50), and Ser675 has been shown to be phosphorylated downstream of p21-activated protein kinase-1 (PAK-1; ref. 42). Hydrolysis of PIP_2 generated by PIPKIγ can induce many physiologic responses depending on the cell type, most notably increasing cellular Ca^{2+} levels (26). As PKA and Ca^{2+} synergize to induce cellular responses, this may explain how PIPKIγ enhances β-catenin activity. In the future, it will be important to further define the kinase(s) responsible for β-catenin phosphorylation downstream of PIPKIγ activity. In addition to playing a potential role in the

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activation of kinases that activate β-catenin, PIP_2 generation might regulate the trafficking of endocytosed vesicles to the lysosome. A dominant-negative PIPKIγ could block E-cadherin degradation, leading to its recycling back to the membrane where it could associate with β-catenin, decreasing its nuclear activity.

The activation of β-catenin by PIPKIγ is almost assuredly through an indirect mechanism. It has yet to be determined whether PIPKIγ activation of β-catenin requires their physical association. Surprisingly, the association between endogenous PIPKIγ and β-catenin decreased upon stimulation of HeLa cells with EGF. This suggests that β-catenin activation by PIPKIγ splice variants was primarily an indirect effect. As endogenous levels of PIPKIγ splice variants localize to different cellular sites, it will be important to further define this interaction to gain insight into its functional role. In addition, further studies are needed to characterize the expression of PIPKIγ splice variants during tumorigenesis. In cells overexpressing PIPKIγ, treatment with EGF enhanced the association between the two proteins. It is plausible that PIPKIγ could localize β-catenin within signaling complexes containing PIP_2 effectors that lead to its activation.

In summary, we have uncovered a novel component within signaling pathways that can be used to activate β-catenin. PIPKIγ expression and activity lead to β-catenin nuclear accumulation, thereby increasing its ability to induce transcriptional changes. Furthermore, we have shown that in response to growth factor stimulation, PIPKIγ expression markedly increases β-catenin activity and that the increase in cell proliferation observed in mesenchymal-like cells overexpressing PIPKIγ requires β-catenin, suggesting a role for this pathway in tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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PIPKIγ Regulates β-Catenin Transcriptional Activity Downstream of Growth Factor Receptor Signaling

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