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Krüppel-like factor 5 incorporates into the β -catenin/TCF complex in response to LPA in colon cancer cells

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Abstract

Lysophosphatidic acid (LPA) is a simple phospholipid with potent mitogenic effects on various cells including colon cancer cells. LPA stimulates proliferation of colon cancer cells by activation of β -catenin or Krüppel-like factor 5 (KLF5), but the functional relationship between these two transcription factors is not clear. Hence, we sought to investigate the mechanism of β -catenin activation by LPA and the role of KLF5 in the regulation of β -catenin by LPA. We found that LPA and Wnt3 additively activated the β -catenin/TCF (T cell factor) reporter activity in HCT116 cells. In addition to phosphorylating glycogen synthase kinase 3 β (GSK-3 β) at Ser9, LPA resulted in phosphorylation of β -catenin at Ser552 and Ser675. Mutation of Ser552 and Ser675 ablated LPA-induced β -catenin/TCF transcriptional activity. Knockdown of KLF5 significantly attenuated activation of β -catenin/TCF reporter activity by LPA but not by Wnt3. However, nuclear accumulation of β -catenin by LPA was not altered by knockdown of KLF5. β -catenin, TCF, and KLF5 were present in a 250–300 kDa macro-complex, and their presence was enhanced by LPA. LPA simulated the interaction of β -catenin with TCF4, and depletion of KLF5 decreased β -catenin-TCF4 association and the transcriptional activity. In summary, LPA activates β -catenin by multiple pathways involving phosphorylation of GSK-3 and β -catenin, and enhancing β -catenin interaction with TCF4. KLF5 plays a critical role in β -catenin activation by increasing the β -catenin-TCF4 interaction.

Keywords

LPA; colon cancer; β -catenin; KLF5; phosphorylation

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1. Introduction

The Wnt/ β -catenin signaling pathway is crucial in the organization and maintenance of the intestinal epithelium [1, 2]. Aberrant β -catenin activation is found in the vast majority of cancers of the gastrointestinal tract [3]. Mutations on adenomatous polyposis loci (*APC*) or β -catenin that result in the activation of Wnt signaling are an early event in the colorectal cancer initiation and progression [4]. When Wnt ligand is absent, the majority of β -catenin is expressed at the cell junction, and the cytoplasmic and nuclear level of β -catenin is kept low. β -catenin is phosphorylated at Ser45 by casein kinase 1 α and subsequently phosphorylated at Ser33, Ser37, and Thr41 by glycogen synthase kinase 3 (GSK-3). Phosphorylated β -catenin is targeted to ubiquitination and proteasomal degradation by the β -catenin destruction complex, which includes APC, GSK-3, and Axin [5]. In the presence of Wnt ligand, phosphorylation at S33/37/T41 is blocked and cytoplasmic β -catenin is stabilized and translocates to the nucleus. In the nucleus, β -catenin activates transcription of downstream target genes such as *c-myc* and *c-jun* through its interaction with the T-cell factor (TCF) and lymphoid enhancer-binding factor (LEF) family of transcription factors [3, 5]. In addition to the N-terminal Ser/Thr residues, Ser552 and Ser675 of β -catenin have also been identified as phosphorylation sites that affect its stability and transcriptional activity [6, 7].

LPA is a small phospholipid molecule that elicits diverse biological effects, including cell survival, proliferation, and migration through a family of G protein-coupled receptors, LPA1–LPA6 [8]. LPA activates multiple signaling pathways, including phosphatidylinositol 3-kinase (PI3K)-Akt, Ras-MEK-ERK, PLC- β -PKC, and Rho-ROCK [9, 10]. Elevated levels of LPA and LPA receptor have been reported in several types of cancers [11, 12].

Transgenic expression of LPA1, LPA2, LPA3, or lysophospholipase D (aka autotaxin) in mouse mammary epithelium resulted in spontaneous metastatic mammary tumors [13]. We have reported the role of LPA2 on intestinal tumor progression such that the absence of LPA2 significantly decreased tumor burden in the *Apc^{Min}* and colitis-associated tumor models of colorectal cancer [14, 15]. A previous report has demonstrated that LPA2 and LPA3 activate the β -catenin pathway in colonic adenocarcinoma HCT116 and LS174T cells, and silencing of β -catenin decreases proliferation of colon cancer cells [16]. Decreased nuclear expression of β -catenin in *Apc^{Min}* intestine by the loss of LPA2 function has corroborated the role of LPA in regulation of β -catenin [15]. LPA does not alter nuclear localization of β -catenin in SW480 and Caco-2 cells that harbor mutations in the *APC* gene and hence β -catenin is constitutively activated [17]. Nevertheless, LPA stimulates proliferation of SW480 and Caco-2 cells despite the lack of effect on β -catenin. We have reported previously that LPA can stimulate colon cancer cell proliferation by induction of Krüppel-like factor 5 (KLF5), a transcription factor highly expressed in the intestinal crypt compartment [17]. While the regulation of β -catenin by LPA is cell line dependent, the induction of KLF5 by LPA appears independent of the mutational status of colon cancer cells. Because silencing of either β -catenin or KLF5 equally blocks colon cancer cell proliferation by LPA, we raise a question whether β -catenin and KLF5 functions independently or in tandem in the presence of LPA. In this study, we investigated the mechanism of β -catenin activation by LPA and the role of KLF5 in the regulation of β -catenin. We found that LPA activates β -catenin by modulating phosphorylation of GSK3

and β -catenin. KLF5 enhances the interaction of β -catenin with TCF4, but KLF5 is not involved in nuclear translocation of β -catenin.

2. Materials and Methods

2.1. Chemicals and antibodies

LPA (18:1, Avanti Polar Lipids, Alabaster, AL) was used at the final concentration of 1 μ M. Recombinant human Wnt3a (R&D Systems, Minneapolis, MN) was used at the final concentration of 200 ng/ml. An equal volume of PBS containing 0.1% BSA was added as a control. LY294002 and H89 were purchased from Sigma. Rabbit anti-KLF5 was a kind gift of Dr. Jonathan Katz (Univ. of Pennsylvania). The following antibodies were purchased: rabbit anti- β -catenin, rabbit anti-non-phospho S33/37/T41 (active) β -catenin, rabbit anti-phospho S552 β -catenin, rabbit anti-phospho S675 β -catenin, rabbit anti-GSK3 α / β , rabbit anti-phospho GSK3 α / β (S21/S9), rabbit anti-TCF4, rabbit anti- β -tubulin, and mouse IgG (Cell Signaling Technology, Danvers, MA); mouse anti- β -catenin (BD Biosciences, Franklin Lakes, NJ); rabbit anti-Lamin-B1 and rabbit anti-KLF5 (Santa Cruz Biotechnology, Paso Robles, CA).

2.2. Cell culture and transfection

HCT116 cells were grown in McCoy's 5A medium, SW480 cells in Roswell Park Memorial Institute (RPMI) 1640, HEK293T and LoVo cells in Dulbecco's modified Eagle's medium (DMEM), all supplemented with 10% fetal bovine serum (FBS), 100 μ g/ml streptomycin, and 100 units/ml penicillin at 37 °C in 95% air, 5% CO₂ atmosphere as previously described [17, 18]. All the cells were serum starved 24 h prior to treatment with LPA or Wnt. Stable knockdown of LPA2 and KLF5 were achieved as described previously [18]. pLKO.1 plasmid harboring small hairpin RNA targeting LPA2 (shLPA2) or KLF5 (shKLF5) was obtained from Sigma-Aldrich (St. Luis, MO). pLKO.1 containing scrambled shRNA, shCont, was used to as a control. Cells transfected with lentivirus were selected by 10 μ g/ml puromycin to obtain stably transfected cells. Silencing of gene products was confirmed by RT-PCR or Western blot.

2.3. DNA Constructs and Mutagenesis

pcDNA3/FLAG- β -catenin was purchased from Addgene (#16828). S552A, S675A, and S552A/S675A mutations in FLAG- β -catenin were made using the QuickChange site-directed mutagenesis kit (Agilent technologies, Santa Clara, CA).

2.4. Luciferase reporter assay

HCT116 cells were transfected with pGL3-TOPflash or pGL3-FOPflash (Promega, Madison, WI) using Lipofectamine 2000 (Life Technologies, Grand Island, NY). pBK-CMV-Renilla luciferase control vector was co-transfected to normalize the transfection efficiency. Cell lysis and reporter assay were performed with the Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. All samples were measured in triplicate or quadruplicate.

2.5. Subcellular fractionation, immunoprecipitation, and Western blot

Isolation of cytoplasmic and nuclear proteins was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Co-immunoprecipitation (Co-IP) of β -catenin, KLF5, and TCF4 was performed as described previously with modifications [19]. Briefly, after collection of cytoplasmic and nuclear protein, protein concentration was determined by the bicinchoninic acid assay (Sigma). Seven hundred μ g of lysate was incubated overnight with or without anti- β -catenin antibody. The immunocomplex was purified by incubation with 70 μ l of protein G-Sepharose beads (GE healthcare) for 1 h, followed by three washes in PBS containing 1% Triton-X, and one wash in PBS. All the above steps were performed at 4°C or on ice. Proteins were eluted by incubating beads in Laemmli sample buffer for 5 min at 95°C. Eluted proteins were separated by SDS-PAGE, and transferred to a nitrocellulose membrane for Western immunoblotting as previously described [20].

2.6. Native PAGE

Nucleus particles were collected using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) as described above. Nuclear lysate was prepared using NativePAGE Sample Prep Kit (Life technologies) with 10% DDM detergent included in the kit according to the manufacturer's instructions. 2 mM dithiobis succinimidyl propionate cross-linker (Pierce) was added to lysate for 30 min on ice, and then quenched by 50 mM Tris HCl for 30 min. For each sample, 8 μ g of nuclear lysate was loaded on NativePAGE Novex 4–16% Bis-Trix Protein Gel. After gel electrophoresis, gel was incubated 1 h in de-ionized water with 1% SDS and 1% β -mercaptoethanol at room temperature. Gel was then transferred to PVDF membrane for Western immunoblotting, as previously described [20].

2.7. Confocal immunofluorescence microscopy

HCT116 cells grown on coverslips were fixed and permeabilized as described previously [18]. Briefly, cells were washed twice with cold PBS, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized in 0.2% Triton X-100 in PBS for 5 min, and blocked in PBS containing 5% normal goat serum for 30 min at room temperature. Cells were co-stained with mouse anti- β -catenin and rabbit anti-KLF5 antibodies for 1 h at room temperature. Following three washes with PBS, for 10 min each, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody and Alexa Fluor 555-conjugated goat anti-mouse antibody for 1 h at room temperature. Nucleus was counterstained with TO-PRO-3 iodide (642/661) (Life technologies), followed by three washes in PBS, for 10 min each. Cells were mounted with ProLong Gold Antifade Reagent (Invitrogen) and observed under a Zeiss LSM510 laser confocal microscope (Zeiss Microimaging, Thornwood, NY).

2.8. Chromatin immunoprecipitation (ChIP) and re-ChIP assay

ChIP was performed using the Magna ChIP G kit (EMD Millipore) as described previously [18]. Briefly, cells were treated with 1% formaldehyde for 15 min to cross-link proteins to DNA, lysed, and then sonicated. The lysate was incubated with TCF4-ChIP validated antibody overnight at 4 °C. The immunocomplex was purified by incubation with 60 μ l of magnetic protein G beads for 1 h and eluted for DNA purification. For re-ChIP assay, 6 IP

reactions were pooled together for one IP. After overnight incubation with TCF4 antibody, the immunocomplex was washed three times with ChIP wash buffer (2mM EDTA, 500mM NaCl, 0.1% SDS, 0.5% Triton-X) and twice with 1×TE buffer, and incubated in 75 µl re-ChIP elution buffer (1×TE, 2% SDS, 15mM DTT, protease inhibitor cocktail) at 37°C for 30 minutes. The immunocomplex was then diluted 20 times with ChIP dilution buffer containing 33 µg/ml BSA and protease inhibitor cocktails, and incubated 2 h with KLF5 antibody at 4°C. The immunocomplex was then washed and DNA was eluted. qRT-PCR was performed with primers for the *c-myc*, *c-jun*, and *lef-1* promoter flanking the putative β-catenin binding sites. Anti-RNA polymerase II and normal mouse IgG were used as the positive and negative control for IP, respectively. The following primers were used for qRT-PCR in ChIP and re-ChIP assay: *c-myc*: 5'-TGCGGGTTACATACAGTGCA-3' and 5'-GCGTCTGTTTAGCCCTGAGA-3'; *lef-1*: 5'-GGAGGAGAAGCAGTGGGGA-3' and 5'-CGAAACGTCCACTTCCTGAA-3'; *c-jun*: 5'-CTCCTCTGTCTGTTGCCCTG-3' and 5'-GCAGGGGCGTTAACATGAAC-3.

2.9. Statistical analysis

Results are presented as the means ± SE. Statistical significance was assessed by a paired *t* test. A *p* value of < 0.05 was considered significant.

3. Results

3.1. LPA and Wnt3a has additive effect on β-catenin accumulation and activity

It has been shown that LPA-mediated cellular signaling pathway cross-talks with the Wnt/β-catenin pathway to inactivate GSK-3 and stimulates nuclear translocation of β-catenin [16]. Fig. 1A shows that LPA increased nuclear expression of β-catenin in HCT116 cells (a 58±13% increase, *p* < 0.05). However, it is not known whether LPA and Wnt ligand cooperatively or independently regulates β-catenin. To address this question, we treated HCT116 cells with Wnt3a, LPA, or both. Activation of β-catenin involves de-phosphorylation at S33/S37/T41 [21]. Western blot shows that LPA and Wnt3a each increased the abundance of active β-catenin (i.e., non-phosphorylated at S33/S37/T41). Exposure of cells to both Wnt3a and LPA further increased the expression level of active β-catenin, indicating that Wnt and LPA additively regulate β-catenin (Fig. 1B). Confocal immunofluorescence analysis in Fig. 1C depicts that LPA and Wnt increased nuclear fluorescence signal of β-catenin, which was augmented by concurrent treatment with LPA and Wnt. The additive nature of β-catenin activation by LPA and Wnt was quantitated by the functional β-catenin/TCF TOPflash reporter assay. The increased reporter activity by LPA or Wnt3a alone was further enhanced by co-treatment with LPA and Wnt3a (Fig. 1D). These results demonstrate that LPA induces β-catenin stabilization, nuclear accumulation, and transcriptional activity independent of Wnt.

3.2. LPA increases phosphorylation of β-catenin and GSK-3

It has been shown previously that LPA-mediated signaling result in phosphorylation GSK-3β at Ser9, which attenuates the activity of GSK-3β towards S33/S37/T41 [16, 22]. Consistently, LPA induced phosphorylation at Ser9 of GSK-3β in HCT116 cells, which was ablated by knockdown of LPA2 (Fig. 2A–B). In addition to the N-terminal phosphorylation

of β -catenin by GSK-3, β -catenin is phosphorylated at Ser552 and Ser675 by Akt and PKA [6, 7]. Since LPA activates several major signaling pathways, including PI3K-Akt and PKC-Rho-ROCK pathways [9, 23], we determined whether treating HCT116 cells with LPA alters β -catenin phosphorylation at Ser552 or Ser675. Under basal conditions, low levels of β -catenin phosphorylation at Ser552 (p-S552) and Ser675 (p-S675) were present in HCT116 cells. LPA increased the levels of p-S552 and p-S675 in a LPA2-dependent manner (Fig. 2A). To determine the signaling pathway regulating phosphorylation of β -catenin, HCT116 cells were treated with LPA in the presence or absence of PKA inhibitor H-89 or PI3K inhibitor LY294002. H-89 partially inhibited LPA-induced phosphorylation at both S552 and S675. Similarly, LY294002 inhibited phosphorylation at S552 and S675 (Fig. 2B). This suggests that phosphorylation of β -catenin at S552 and S675 by LPA is partially dependent on both PKA and PI3K.

Phosphorylation of β -catenin at Ser552 and Ser675 causes its disassociation from cell-cell contact and nuclear accumulation [6, 7, 24]. Hence, we next examined whether β -catenin phosphorylation at Ser552 and Ser675 affects its subcellular distribution. Western blot of cytoplasmic and nuclear extracts showed that LPA significantly increased (~ 3 fold) the expression levels of p-S552 and p-S675- β -catenin in the nucleus (Fig. 2C).

To determine the functional importance of phosphorylation at Ser552 and Ser675 by LPA, we changed Ser552 and Ser675 to Ala individually or together. These constructs were transiently expressed in HCT116 cells to assess their effects on the β -catenin/TCF transcriptional activity. LPA-mediated stimulation of TOPflash reporter activity was blunted by exogenous expression of S552A, S675A, or S552A/S675A (AA) mutations. These results suggest that phosphorylation at Ser552 and Ser675 by LPA facilitates β -catenin nuclear translocation and increases transcriptional activity. Moreover, a single Ser-to-Ala mutation did not differ from double mutations, indicating that mutation at one Ser site is sufficient to block β -catenin nuclear accumulation by LPA.

3.3. KLF5 is not necessary for β -catenin nuclear accumulation by LPA

It was shown recently that KLF5 interacts with β -catenin to enhance nuclear translocation of β -catenin in COS-1 cells [25], but whether β -catenin and KLF5 are functionally associated in the context of LPA signaling has not been determined. To address this question, HCT116 cells were stably transfected with shKLF5 or scrambled shCont (Fig. 3A). Knockdown of KLF5 did not affect phosphorylation of β -catenin or GSK-3 β by LPA (Fig. 3B). LPA induced KLF5 (green, Fig. 3C) and β -catenin (red) expression in the nuclei of shCont transfected cells (Fig. 3C). Surprisingly, silencing of KLF5 did not alter nuclear accumulation of β -catenin by LPA. This observation was confirmed by Western blot of the nuclear extract, which showed increased β -catenin expression in both control and KLF5 knockdown cells (Fig. 3D). These results indicated that KLF5 does not regulate LPA-induced nuclear accumulation of β -catenin.

3.4. β -catenin, KLF5, and TCF4 form a complex and the interaction is increase by LPA in nuclei

Once β -catenin translocates into the nucleus, it binds TCF/LEF transcription factors to activate gene expression [3, 5]. Although KLF5 did not regulate nuclear translocation of β -catenin, we sought to determine whether KLF5 affects the β -catenin/TCF transcriptional activity. Knockdown of KLF5 blocked LPA-induced β -catenin/TCF4 transcription activity (Fig. 4A). On the contrary, stimulation of the β -catenin/TCF4 activity by Wnt3a was not affected by KLF5 knockdown. In line with data shown in Fig. 1, concurrent treatment with LPA and Wnt3a further increased β -catenin/TCF reporter activity. Importantly, silencing of KLF5 decreased the TOPflash reporter activity to the level attained by Wnt3a alone. These results corroborate the importance of KLF5 in LPA-mediated regulation of β -catenin/TCF transcription activity.

Because our results indicate that KLF5 is involved in regulation of β -catenin/TCF activity, we sought to determine whether β -catenin interacts with KLF5 in the nucleus. To this end, we performed co-IP of β -catenin and KLF5 in HCT116 cell nuclear extract. Co-IP was performed using 0.5 μ g of anti- β -catenin antibody. Under this condition, we presumed that the amount of antibody is the limiting factor so that the equivalent amount of β -catenin will be immunoprecipitated from all samples. Indeed, the amount of immunoprecipitated β -catenin did not significantly change supporting our assumption (Fig. 4B). Fig. 4B shows that TCF or KLF5 was not co-immunoprecipitated with β -catenin from the cytoplasm although their interaction might have been below the limit of detection by Western blot. On the contrary, co-IP of TCF4 and KLF5 in the nuclear fraction was evident under basal condition, and LPA enhanced their interaction. Mock IP with mouse IgG did not pull-down TCF4 or KLF5, confirming the specificity of co-IP (Fig. 4C). These results suggest that, in addition to TCF, β -catenin interacts with KLF5 in the nucleus. Since β -catenin and TCF form a transcription complex, we contemplate that KLF5 is present in the same complex. To determine the presence of β -catenin, KLF5, and TCF4 in the same complex, we resolved the nuclear extract of HCT116 cells by native PAGE. As shown in Fig. 4D, β -catenin was present primarily in a multi-protein complex with molecular mass of 250–300 kDa and in a 500–600 kDa complex by lesser extent. The presence of KLF5 and TCF4 in the 250–300 kDa complex was readily detected, but we could not detect their presence in the larger complex even after prolonged exposure. In addition, the expression levels of β -catenin, KLF5, and TCF4 in the 250–300 kDa complex were significantly increased after LPA treatment.

We next inquired whether LPA-induced interaction between β -catenin and TCF4 is specific to HCT116 cells or occurs in other colon cancer cells. To this end, we performed co-IP in LoVo and SW480 cells. SW480 cells have a truncating *APC* mutation at codon 1338 and a complete loss of the second allele, while LoVo cells have a heterozygous deletion of *APC* [26, 27]. Fig. 4E shows that LPA increased KLF5 expression in both LoVo and SW480 cells, consistent with our previous report that KLF5 induction by LPA is independent of APC/ β -catenin [17]. LPA stimulated nuclear accumulation of β -catenin in LoVo, but we could not observe a significant change in SW480 cells. The lack of effect on nuclear expression of β -catenin in SW480 cells is consistent with our earlier study [17].

Nonetheless, LPA significantly increased the efficacy of KLF5 or TCF4 co-immunoprecipitating with β -catenin in both LoVo and SW480 cells.

3.5. KLF5 is critical for the LPA-induced interaction between β -catenin and TCF4, and β -catenin activation

Since LPA enhances the interaction between β -catenin and TCF4, we asked whether KLF5 plays a role in the regulation of β -catenin-TCF4 interaction. As shown in Fig. 5A, LPA stimulated co-IP of KLF5 and TCF4 with β -catenin in control transfected cells. Knockdown of KLF5 did not appear to have a significant effect on co-IP under basal conditions. However, LPA failed to increase β -catenin-TCF4 interaction with KLF5 knockdown, suggesting that KLF5 is necessary for the interaction between β -catenin and TCF4 by LPA.

To determine the functional significance of KLF5 in β -catenin/TCF4 transcriptional activity, we assessed β -catenin/TCF4 downstream target gene expression by ChIP assays. Fig. 5B shows that LPA stimulated binding of the *c-jun*, *c-myc*, and *lef-1* promoters to TCF4, which was ablated by depletion of KLF5. To confirm the presence of KLF5 in the transcriptional machinery when the β -catenin/TCF4 complex binds to the promoters, we performed re-ChIP assay by sequential IP of TCF4 and KLF5, followed by qRT-PCR. Fig. 5C shows the presence of the *c-jun*, *c-myc*, and *lef-1* promoters in the KLF5-immunocomplex. Moreover, KLF5 silencing significantly decreased the occupancy of the *c-jun*, *c-myc* and *lef-1* promoters. These results indicate that KLF5 is a component of the transcription complex containing β -catenin and TCF4.

In Fig. 4D, LPA did not alter nuclear β -catenin abundance in SW480 cells and yet the interaction with KLF5 was increased. We assessed whether β -catenin/TCF4 transcriptional activity in SW480 cells is altered by LPA. In SW480 cells, the basal TOPflash reporter activity was markedly elevated compared with HCT116 cells (not shown), and yet LPA resulted in statistically significant increase in the reporter activity. Importantly, depletion of KLF5 mitigated the change, demonstrating that KLF5 promotes the β -catenin/TCF4 transcriptional activity in cells with constitutively active Wnt/ β -catenin signaling (Fig. 5D). Moreover, increased β -catenin phosphorylation was observed in SW480 cells in response to LPA (Fig. 5E). Together, these results demonstrate that LPA-induced KLF5 regulates the activity of β -catenin/TCF4 complex by enhancing the interaction between β -catenin and TCF4, which subsequently affects the occupancy of the TCF4 target gene promoters.

4. Discussion

The activity of Wnt signaling hinges on the expression, stability and nuclear translocation of β -catenin. The study by Yang et al. [16] has shown that LPA stimulates colon cancer cell proliferation by inducing nuclear translocation of β -catenin. Absence of LPA2 decreases β -catenin expression in adenomas in mouse intestine [15]. Previous study has shown that LPA-induced phosphorylation of GSK-3 β is PKC-dependent [16], but how LPA stimulates β -catenin is not well known. The findings in the current study show that activation of β -catenin by Wnt and LPA is not redundant but additive. We show that LPA regulates β -catenin through multiple mechanisms and KLF5 functions as a co-factor, which facilitates

the association of β -catenin with TCF4 and thereby increases the transcriptional activity of TCF4.

The Wnt/ β -catenin pathway is frequently mutated in many cancers including colorectal cancer, and yet LPA stimulates proliferation of cancer cells harboring a mutation. For instance, LPA induces proliferation of SW480 cells that have mutations in *APC* [17]. We have previously shown that KLF5 is important for LPA-mediated proliferation of colon cancer cells and have suggested that KLF5-dependent regulation as an alternative mechanism of cell proliferation. However, silencing of either β -catenin or KLF5 almost completely blocks LPA-dependent cell proliferation [16, 17]. In this study, we investigated the mechanism of β -catenin activation by LPA and the functional relationship between β -catenin and KLF5.

Phosphorylation represents a key mechanism responsible for the tight control of β -catenin expression and activation of the Wnt/ β -catenin pathway. ERK phosphorylates GSK-3 β at Thr43 that primes GSK-3 β for phosphorylation at Ser9 [28]. In agreement with previous studies [16, 22], LPA phosphorylated GSK-3 β at Ser9 and increased the expression level of non-phosphorylated β -catenin. In addition, we found that LPA phosphorylated β -catenin at Ser552 and Ser675. Previous studies have shown that phosphorylation at Ser552 is mediated by Akt, whereas PKA is responsible for phosphorylation at Ser552 and Ser675 [6, 7, 24]. Interestingly, inhibition of PI3K by LY294002 or PKA by H-89 blocked LPA-induced phosphorylation at both Ser552 and Ser675. The dual and partial effects of each inhibitor suggest that it is unlikely that either Akt or PKA is directly involved in phosphorylation of β -catenin by LPA. The effect of H-89 is interesting since LPA receptors, with an exception of LPA5, are shown to inhibit cAMP accumulation [29]. Although H-89 is often used as a non-specific inhibitor of PKA, it is known to inhibit at least 8 other protein kinases, including Akt and Rho-dependent protein kinase [30]. Moreover, a recent study has shown that p21-activated kinase 1 (PAK1) interacts and phosphorylates β -catenin at S675 [31]. Additionally, PI3K activates PAK1 [32], implying that PAK1 may be involved in LPA-mediated phosphorylation of β -catenin. Although PAK1 regulation by LPA was not determined in our current study, evidence shows PAK1 activation by LPA [33, 34]. Phosphorylation of β -catenin at Ser552 and Ser675 results in increased nuclear translocation and transcriptional activity [6, 7, 24]. Consistently, we found that LPA increased the level of p-S552 or p-S675 β -catenin in the nucleus. The replacement of the Ser residues to Ala decreased nuclear abundance of β -catenin and β -catenin/TCF transcriptional activity, demonstrating LPA facilitates β -catenin activity by phosphorylation of β -catenin.

KLF5 is highly expressed in the intestine, particularly enriched in the proliferating crypt cell population where it positively regulates cell proliferation [35]. Intestinal *Klf5* deletion in mice disrupts canonical Wnt signaling [36], and KLF5 haploinsufficiency suppresses adenoma formation in *Apc^{Min}* mice with decreased expression of β -catenin and the Wnt target *myc* [25]. We have shown previously that LPA induces KLF5 expression in colon cancer cells [17]. This induction is independent of the mutational status of the Wnt/ β -catenin pathway, K-Ras, or p53 [17, 37]. Silencing of KLF5 markedly inhibits proliferation of SW480 cells in which β -catenin nuclear expression does not respond to LPA [17]. These results appear to suggest that LPA regulates colon cancer cells by at least two independent

pathways involving β -catenin or KLF5. McConnell *et al.* [25] showed recently that KLF5 interacts with β -catenin to facilitate nuclear translocation of β -catenin in COS-1 cells, suggesting the possibility of mechanistic overlap between β -catenin and KLF5. However, we could not observe a significant difference in nuclear expression of β -catenin with silencing of KLF5. The reason for the discrepancy is not clear, but this may be attributed to the different amounts of β -catenin and/or KLF5 in COS-1 and HCT116 cells. In the former study, β -catenin and KLF5 were transfected in COS-1 cells, which probably led to higher expression of these proteins than in the cell lines examined in the current study. Although KLF5 did not show an apparent effect on nuclear targeting of β -catenin, we observed increased interaction between KLF5 and β -catenin in the nucleus. Our results show that β -catenin, TCF4, and KLF5 are part of a large complex with apparent molecular mass of >250 kDa, and that LPA increases their presence in the complex. In addition to increasing the interaction of β -catenin with KLF5, LPA enhanced the interaction of β -catenin and TCF4. The increased protein association was observed in HCT116, LoVo, and SW480 cells. This effect in SW480 cells is particularly interesting since these cells harbor mutations that delete APC [26]. With constitutively active Wnt/ β -catenin pathway, the nuclear level of β -catenin was not altered by LPA. Yet, we observed increased interaction of β -catenin with KLF5 and TCF4, and a concurrent increase in the β -catenin/TCF transcriptional activity. The effect in SW480 cells and the additive effect of LPA and Wnt3a are in principle consistent, both demonstrating the positive role of LPA in regulation of Wnt/ β -catenin pathway.

Our recent study showed that KLF5 binds the *hif1* promoter to compete out p53 and induce HIF-1 α expression [37]. Because knockdown of KLF5 decreased the association of β -catenin and TCF, we propose a new function of KLF5 as a cofactor for the β -catenin/TCF transcription complex. However, the mechanism by which KLF5 facilitates the β -catenin-TCF association is not yet clear. Unlike co-regulators such as cAMP response element binding protein (CBP) or p300, KLF5 lacks protein interaction domains. However, it was shown that KLF5 binds CBP/p300 and it is acetylated at defined sites [38, 39]. It was additionally shown that phosphorylation at the N-terminal region of KLF5 promotes its transactivation function [40]. Hence, it is conceivable that post-translational modification, including phosphorylation or acetylation, enhances the interaction of β -catenin with KLF5 although such a change in the context of LPA signaling is yet to be determined. Despite the evidence that KLF5 increases the efficacy of transcription by the β -catenin/TCF complex, one question remains to be evaluated is whether the incorporation of KLF5 alters the target specificity of the β -catenin/TCF complex.

In summary, this study describes the mechanism by which LPA-elicited signaling regulates the Wnt/ β -catenin pathway. It shows that LPA intersects the Wnt/ β -catenin pathway at multiple points. The N-terminal phosphorylation of GSK-3 β , phosphorylation of β -catenin at S552 and S675, and the induction of KLF5 collectively activate β -catenin. KLF5 does not alter nuclear translocation of β -catenin but functions as a co-regulator of the β -catenin/TCF transcription complex to augment its transcriptional activity.

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Abbreviations

LPA	lysophosphatidic acid
KLF5	Krüppel-like factor 5
TCF	T-cell factor
LEF	lymphoid enhancer-binding factor
APC	adenomatous polyposis loci
GSK	glycogen synthase kinase
PI3K	phosphatidylinositol 3-kinase
IP	immunoprecipitation
ChIP	chromatin immunoprecipitation
PAK1	p21-activated kinase 1
CBP	cAMP response element binding protein

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Highlights

- LPA and Wnt3 additively activate β -catenin
- LPA increases phosphorylation of β -catenin at S552 and S675
- KLF5 is required for β -catenin activation by LPA but for nuclear accumulation of β -catenin
- β -catenin, KLF5 and TCF4 form a complex in the nucleus
- KLF5 facilitates LPA-induced interaction between β -catenin and TCF4

Figure 1. LPA and Wnt3a additively induce β -catenin accumulation and activity
(A) HCT116 cells were treated with LPA for 4 hours, and whole cell lysate, cytoplasmic, and nuclear extracts were collected. Expression levels of β -catenin were determined by Western blot. β -tubulin was used as a cytoplasmic marker. Representative figures from three independent experiments are shown. Relative changes were quantified by densitometric analysis of three independent experiments and expressed as percent changes relative to conditions without LPA. **(B)** HCT116 cells were treated with LPA (1 μ M), Wnt3a (100 ng/ml), or both for 4 hours. Activation of β -catenin was determined by assessing active, non-phosphorylated β -catenin expression. Whole cell lysate was used for Western blotting using an antibody specific to nonphospho-S33/S37/T41 β -catenin. **(C)** Cellular localization of β -catenin (red) was determined by confocal immunofluorescence microscopy in cells treated with LPA, Wnt3a, or both for 4 hours. TO-PRO was used for nuclear counterstaining (magenta). **(D)** TOPflash construct were used to evaluate the β -catenin/TCF transcriptional

activity in cells treated with LPA, Wnt3a, or both. FOPflash was used as a negative control for TOPflash. The luciferase activity was normalized against *Renilla* activity. Data (means $\pm$ SE) presented are the relative luciferase activity of three independent experiments. *, $p < 0.05$. n=3.

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Figure 2. LPA increases phosphorylation of β-catenin at Ser552 and Ser675

(A) HCT116 cells transduced with shCont or shLPA2, and LPA2 knockdown efficiency was evaluated by qRT-PCR. (B) HCT116 cells transduced with shCont or shLPA2 were treated with LPA for 15 min, and phosphorylation of GSK-3β (Ser9) and β-catenin (Ser552 and Ser675) was determined. β-actin was used as a loading control. All figures are representatives of at least three experiments. Relative phosphorylation levels were quantified by densitometric analysis and expressed as percent changes relative to cells without LPA under each condition. (C) Cells were pretreated with H89 (10 μM) or LY294002 (10 μM) for 30 min prior to stimulation by LPA for 15 min. Phosphorylation of β-catenin was determined as described above. Protein band intensity is expressed as a percent change relative to control without LPA. n=3. (D) Expression levels of p-S552 and p-S675 β-catenin were determined in cytoplasmic and nuclear fractions. β-tubulin and Lamin B1 were used as cytoplasmic and nuclei loading control, respectively. (E) Cells transiently transfected with FLAG-tagged WT, S552A, S675A, or S552A/S675A (AA) β-catenin were treated with LPA for 4 h. The β-catenin/TCF transcriptional activity was assayed using TOPflash as described above. Data (means ± SE) presented are the relative luciferase activity of three independent experiments. *, $p < 0.01$. **, $p < 0.05$. n=3.

Figure 3. KLF5 is not required for β-catenin phosphorylation or nuclear accumulation
 (A) Induction of KLF5 by LPA and KLF5 knockdown efficiency are shown. (B) The effect of KLF5 knockdown on phosphorylation of β-catenin (Ser552 and Ser675) and GSK-3β (Ser9) was determined. (C) Cellular localization of β-catenin (red) and KLF5 (green) in cells transduced with shCont and shKLF5 cells were evaluated by confocal immunofluorescence microscopy. TO-PRO was used for nuclear counterstaining (magenta). (D) Nuclear fractions of shKLF5 and shCont transduced cells were collected and the expression levels of β-catenin was determined. Lamin B1 was used as a loading control. All data are representatives of at least three independent experiments.

Figure 4. KLF5 interacts with β-catenin in the nucleus

(A) HCT116 cells transduced with shCont and shKLF5 were treated with LPA for 4h, and β-catenin/TCF reporter assay was performed. Data represent relative luciferase activity (means ± SE) of three independent experiments. *, $p < 0.01$. n=3. (B) β-catenin was immunoprecipitated from HCT116 nuclear extract, followed by Western blot for TCF4 or KLF5. Lower panels show protein expression in cytoplasmic and nuclear lysate. (C) HCT116 nuclear extract was immunoprecipitated with anti-β-catenin antibody (+) or mouse IgG (-) as a mock control, followed by Western blot for TCF4 or KLF5. Left lane (input)

shows protein expression in nuclear lysate. **(D)** Nuclear extract from cells treated with or without LPA for 4 h was resolved by native PAGE and the expression of β -catenin, KLF5 and TCF4 was determined. **(E)** Co-IP of TCF4 and KLF5 with β -catenin was performed in nuclear extracts of LoVo and SW480 cells. Lower panels show protein expression in nuclear lysate. Data are representative of three independent experiments.

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Figure 5. Enhanced interaction between β -catenin and TCF4 by LPA requires KLF5

(A) β -catenin was immunoprecipitated from nuclear extracts of shCont and shKLF5 transduced HCT116 cells. The presence of TCF4 and KLF5 in the immunoprecipitates was determined. Lower panels show expression of β -catenin, KLF5 and TCF4 in the nuclear extract. Lamin B1 was used as a loading control. (B) Cells were fixed with formaldehyde, and cell lysates were immunoprecipitated with anti-TCF4 antibody, followed by qRT-PCR to detect bound *c-jun*, *c-myc* and *lef-1* promoter DNA fragments. Grey bars, shCont; dark bars, shKLF5. *, $p < 0.05$. $n=3$. (C) The presence of KLF5 in the β -catenin/TCF4 transcription complex determined by re-ChIP assay as described in Materials and Methods. Sequential immunoprecipitation was performed using TCF4 and KLF5 antibody, followed by qRT-PCR to quantify the bound promoter DNA fragments. *, $p < 0.05$. $n=3$. (D) The β -catenin/TCF4 reporter activity was determined in SW480 cells treated with shCont or shKLF5. Grey bars, control; dark bars, LPA. *, $p < 0.05$. $n = 3$. (E) β -catenin phosphorylation in SW480 cells treated with or without LPA was determined. $n = 2$.