Protein Kinase C α but not PKCζ Suppresses Intestinal Tumor Formation in *Apc*$^{Min/+}$ Mice

Henrik Oster[1] and Michael Leitges[1,2]

[1]Laboratory for Signal Transduction, Max Planck Institute of Experimental Endocrinology and [2]Department of Nephrology, Hannover Medical School, Hannover, Germany

Abstract

Members of the protein kinase C (PKC) family of serine/threonine kinases play key regulatory roles in numerous cellular processes, including differentiation and proliferation. Of the 11 mammalian PKC isoforms known, several have been implicated in tumor development and progression. However, in most cases, isotype specificity is poorly defined, and even contrary functions for a single PKC have been reported mostly because appropriate molecular and genetic tools were missing to specifically assess the contribution of single PKC isoforms *in vivo*. In this report, we therefore used *PKC* genetic targeting to study the role of PKCα and PKCζ in colorectal cancer. Both isoforms were found to be strongly down-regulated in intestinal tumors of *Apc*$^{Min/+}$ mice. A deletion of *PKCζ* did not affect tumorigenesis in this animal model. In contrast, *PKCα*-deficient *Apc*$^{Min/+}$ mice developed more aggressive tumors and died significantly earlier than their PKCα-proficient littermates. Even without an additional *Apc* mutation, *PKCα knockout* mice showed an elevated tendency to develop spontaneous intestinal tumors. Transcriptional profiling revealed a role for this kinase in regulating epidermal growth factor receptor (EGFR) signaling and proposed a synergistic mechanism for EGFR/activator protein and WNT/APC pathways in mediating intestinal tumor development. (Cancer Res 2006; 66(14): 6955-63)

Introduction

Colorectal cancers constitute the second most fatal class of malignant diseases responsible for ~10% of all cancer-related deaths (>50,000 cases in 2005) in the United States (1). The risk to acquire colorectal cancer is heavily influenced by lifestyle, but about 20% to 30% of all cases can be attributed to genetic predisposition; 3% to 5% of all patients derive from genetically characterized high-risk families (2). Additionally, about 80% of all forms of sporadic colorectal cancer are characterized by one or more mutations in the *adenomatous polyposis coli* (*Apc*) gene, of which about 60% result in the expression of a truncated version of the corresponding protein (3). APC forms a multimeric complex with the adaptor protein AXIN2 and glycogen synthase kinase 3β (GSK-3β), which regulates nuclear accumulation of β-CATENIN, a signal transducer of the WNT pathway (4). When the APC/β-CATENIN complex is destabilized due to *Apc* mutations, β-CATENIN binds and activates transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family that regulate the expression of potent oncogenes like *c-Myc* or *c-Met* (5).

The protein kinase C (PKC) family of serine/threonine kinases comprises at least 11 isozymes in mammals. According to structural and activation criteria, PKCs are divided into three subfamilies: classical (or conventional) PKCs [cPKC: PKCα, PKCβ (comprising the two splice variants β1 and β2), and the neuron-specific PKCγ], novel PKCs (nPKC: PKCδ, PKCε, PKCη, and PKCθ), and atypical PKCs (aPKC: PKCι/λ, PKCζ, and PKMζ), an alternatively transcribed, constitutively active variant of PKCζ, the expression of which is restricted to the central nervous system and the spinal cord (6). The activation of cPKC and nPKC isoforms typically involves recruitment to membranes and interaction with and allosteric activation by diacylglycerol. For aPKCs, activation can be driven in part by interaction with the CDC42/GTP/PAR6 complex, which binds to the PB1 domain of aPKCs. In each case, the allosteric effects of these lipids/proteins on PKC isoforms lead to a loss of the inhibition exerted by an inhibitory pseudosubstrate sequence that otherwise occupies the catalytic site of the enzyme (7).

PKCs have been shown to participate in signaling pathways regulating a plethora of biological functions, including cell growth, differentiation, apoptosis, transformation, and tumor development (8). There is evidence that the wide spectrum of PKC-mediated signaling is organized by isotype specificity that despite the broad overlapping substrate specificities is defined via unique expression patterns, intracellular localization, and specific binding partners (9). The initial observation that PKCs can be activated by phorbol esters suggested a role for these enzymes in tumor promotion (10). However, with the development of more selective pharmacologic inhibitors and of molecular and genetic tools to directly target single PKC isoforms, distinct and sometimes opposing roles have been shown for different PKCs in cell growth and differentiation (11). Even the action of one single isotype may vary with different cell types or *in vivo* systems studied. For example *PKCα* has been proposed to act both as tumor promoter and a tumor suppressor in intestinal cancer via multiple signaling pathways (12).

To further clarify the role of different *PKC* isoforms in the development and progression of colorectal cancer *in vivo*, we studied the consequences of *PKC* genetic depletion on tumor development in the *Apc*$^{Min/+}$ (multiple intestinal neoplasia) mouse model. *Apc*$^{Min/+}$ animals harbor a nonsense mutation at codon 850 of the *Apc* gene (13). Although homozygous animals die before birth, all heterozygous mice develop multiple adenomas throughout the entire intestinal tract at an early age (14), which made them a widely used model for studying colorectal cancer biology.

In this study, we show that of six PKC isoforms significantly expressed in the *Apc*$^{Min/+}$ intestine, *PKCα* and *PKCζ* are down-regulated in tumor tissues. Loss of *PKCα* increases tumor numbers, promotes adenoma to adenocarcinoma transition, and reduces

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Michael Leitges, Department of Internal Medicine/Nephrology, Hannover Medical School, Carl Neuberg Strasse Nr. 1, Hannover, Germany 30625. Phone: 49-511-5359-127; Fax: 49-511-5359-186; E-mail: michael.leitges@mpihan.mpg.de.

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survival rates in $Apc^{Min/+}$ mice, whereas deletion of *PKCζ* does not affect intestinal carcinogenesis in this model. Already in the absence of an *Apc* mutation, a *PKCα* deficiency favors intestinal polyp formation. Transcriptional profiling revealed that the epidermal growth factor (EGF) *Betacellulin* (*Btc*) is up-regulated in the absence of *PKCα*, and that EGF and WNT signals may synergistically promote cancer development via shared transcriptional targets.

Materials and Methods

Animals. C57BL/6J-$Apc^{Min/+}$ and wild-type C57BL/6J animals were purchased from The Jackson Laboratory (Bar Harbor, ME). $PKC\alpha^{-/-}$ (15) and $PKC\zeta^{-/-}$ (16) mice were kept on a 129S2/Sv background. Most of the $APC^{Min/+}$/PKC–deficient animals were on a mixed 129S2/Sv × C57BL/6J background. For polyp/tumor scoring and the establishment of survival curves, all animals received high nutrient food (Teklad #2019 extruded, Harlan Winkelmann, Borchen, Germany) and water *ad libitum*, whereas mating pairs were fed on standard mouse chow (ssniff M-Z, sniff Spezialdiäten, Soest, Germany).

***In situ* hybridization.** *In situ* hybridization (ISH) using ^{35}S-UTP-labeled RNA probes was done on 7-μm sections from paraformaldehyde-fixed, paraffin-embedded tissue samples as described (17). The primers used to PCR amplify PKC cDNA templates from reverse transcribed E14.5 embryo total RNA preparations were as follows: *PKCα*, 5′-CCATCAGTGGGAAGTGATCC-3′ and 5′-TGGTTGTGCTATGATGACTG-3′; *PKCβ*, 5′-CCCACATCGACAGAGAGGTT-3′ and 5′-AGTTTCATCCGGTCCCTGTT-3′; *PKCγ*, 5′-TCTGCAGTCACTGTACCGACTT-3′ and 5′-GCTTTCACGGTCTTTGTCTTCT-3′; *PKCδ*, 5′-GCGAGGGAAGACACTGGTAC-3′ and 5′-CGGCCGATAATCTTGTCAAT-3′; *PKCε*, 5′-TTGGACCCCTACATTGCCCTTAAC-3′ and 5′-TGTAGTTGTGGATCCCGAAC-3′; *PKCη*, 5′-GCACTCGCTCTTCAAAAAGG-3′ and 5′-GCAAGTGCAGGCTGTAACAA-3′; *PKCθ*, 5′-CTTGTCAGGGAGAGGCAGTG-3′ and 5′-ATCGCTGCATTACACTGTCG-3′. Probes for *PKCι/λ* and *PKCζ* were as described (6). After hybridization and washing, slides were dipped in Kodak NTB-2 emulsion (Kodak, Stuttgart, Germany) and counterstained with Hoechst dye (Sigma-Aldrich, Seelze, Germany). Artificially colored dark field and blue fluorescence micrographs of the same area were captured on a Leica DMR microscope (Leica Microsystems, Bensheim, Germany) using an Olympus DP50 CCD camera (Olympus, Hamburg, Germany) and overlaid using Photoshop software (Adobe, San Jose, CA).

Quantitative PCR. Tumor and adjacent normal tissue samples were dissected from PBS-rinsed, methylene blue–stained intestinal preparations under a dissecting microscope. Total RNA was extracted using the QIAGEN RNeasy micro kit (Qiagen, Hilden, Germany) and reverse transcribed with Thermoscript RT (Invitrogen, Paisley, United Kingdom). Quantitative PCR was done on an iCycler thermal cycler (Bio-Rad, Hercules, CA) with iQ SYBR Green supermix (Bio-Rad) according to the manufacturer's protocols. Primers used were as follows: *Ef1α*, 5′-AATTCACCAACACCAGCAGCAA-3′ and 5′-TGCCCCAGGACACAGAGACTTCA-3′; *PKCα*, 5′-GCCCGAGGGAGAGAGGGATG-3′ and 5′-TGGGGAGGCATTTGATCTTTCATTT-3′; *PKCβ*, 5′-CCGGTGTGGATGGCTGGTTC-3′ and 5′-GCCTTTCCCCAGCACCATCA-3′; *PKCγ*, 5′-CTTTGGTGCCAGCCCAGGAC-3′ and 5′-CAGGACCTCGGCCTCCCAAT-3′; *PKCδ*, 5′-TGCAGCATCAGGCCAAGGTG-3′ and 5′-CAGCGTTGCATTGCCTGCAT-3′; *PKCε*, 5′-TCCCTGCTCTGGGGCCTCTT-3′ and 5′-GGTGCCGATCCTAAACCCAGGA-3′; *PKCη*, 5′-CGCCAGTTAGAGCCGCCTTTC-3′ and 5′-GGCTACAGTTGCAATTCCGGTGA-3′; *PKCθ*, 5′-GCAGGGTTACCAGTGCCGACAG-3′ and 5′-TCGGTGGTGGACGTTCATGC-3′; *PKCι/λ*, 5′-AGGAACGATTGGGTTGTCAC-3′ and 5′-AGGAACGATTGGGTTGTCAC-3′; *PKCζ*, 5′-GCCTCCCTTCCAGCCCCAGA-3′ and 5′-CACGGACTCCTCAGCAGACAGCA-3′; *Btc*, 5′-TGCCACGCTGGGTCCTATTT-3′ and 5′-CCAAACCGATCCCTGCACTC-3′; *Jun*, 5′-GCATGAGGAACCGCATTGC-3′ and 5′-GCCACCTGTTCCCTGAGCAT-3′; *Axin2*, 5′-AAGAGAAGCGACCCAGTCAATC-3′ and 5′-AGCTGTTTCTTACTCCCCATGC-3′. Single well amplification efficiency estimation and relative quantification of expression levels were done as described (18).

Intestinal polyp scoring. Mice of the specified age were sacrificed by cervical dislocation, and whole intestines were immediately removed and incubated with ice-cold PBS. Small intestines were divided into three sections of equal length ("proximal," "medial," and "distal"), and colons (including the proximal part of the rectum) were separated from the *cecum* and the rectal opening. All sections were carefully rinsed with ice-cold PBS, split longitudinally, and mounted between two glass slides. Tumors/polyps were counted after paraformaldehyde fixation and methylene blue staining under a dissecting microscope using 10-fold magnification.

Histologic analysis and immunohistochemistry. Intestinal samples were split longitudinally and fixed with paraffin in a Swiss roll preparation at 4°C overnight. After dehydration and paraffin embedding, 7-μm sections were either used for Harris' H&E-xylene histologic staining (protocol adopted from http://science.peru.edu/gregarina/html/harris.html) or submitted to immunohistochemical staining. Primary rabbit anti-phospho-extracellular signal-regulated kinase 1/2 (anti-phospho-ERK1/2; pT185/pY187; Biomol, Matford Court, United Kingdom) and anti-ERK1/2 (Stressgen, San Diego, CA) antibodies were used at a 1:200 dilution. Primary rabbit anti-phospho-EGF receptor (anti-phospho-EGFR; pY1086, Biosource, Camarillo, CA), anti-phospho-histone H3 (pS10, Upstate, Charlottesville, VA), and goat anti-BTC (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used at 1:100 dilution. Biotin/avidin amplification (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine/nickel staining were done as described (17).

Microarray analysis. Affymetrix MG 430A 2.0 mouse genome arrays (Affymetrix, Santa Clara, CA) were hybridized with biotin-labeled cRNA samples from total RNA preparations (QIAGEN RNeasy micro kit) from wild-type and $PKC\alpha^{-/-}$ small intestine samples (s.a.) at the RZPD Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany. Sample normalization (PM/MM model), probe set comparison (mutant versus wild-type signal: >1.5 or <0.67, lower 90% confidence bound; signal difference > 50) and hierarchical clustering were done using dChip 1.3 software (19). All array data have been submitted to the National Center for Biotechnology Information GEO data base (accession nos. GSM89632 to GSM89634 and GSE3915).

Results

***PKCα* and *PKCζ* are down-regulated in $Apc^{Min/+}$ intestinal tumors.** To specifically investigate the regulation of *PKC* expression in the $Apc^{Min/+}$ mouse model, we used isoform-specific ISH and quantitative real-time PCR approaches. Of nine isoforms tested [*α, β* (including both *β1* and *β2*), *γ, δ, ε, η, θ, ι/λ*, and *ζ*], only six (*α, β, δ, η, ι/λ*, and *ζ*) were detected by ISH in the murine intestine (Fig. 1*A*). Comparison between tumor and normal tissue signals revealed a strong up-regulation of *PKCβ, PKCι/λ* and, to a lesser extent, *PKCη* as well as a significant down-regulation of *PKCα* and *PKCζ*, whereas *PKCδ* expression was not regulated. To confirm these findings and quantify the expression changes in the regulated transcripts, we repeated these measurements by quantitative PCR on total RNA extractions from $Apc^{Min/+}$ tumors and adjacent normal tissue samples (Fig. 1*B*). In this assay, *PKCβ* was most strongly up-regulated (10.5-fold) followed by *PKCι/λ* (4.6-fold) and *PKCη* (1.7-fold). Again, *PKCα* and *PKCζ* appeared down-regulated in tumors (7.6- and 5.5-fold, respectively), whereas all other isoforms were not differentially expressed.

***PKCα* but not *PKCζ* deficiency enhances tumor formation in the intestine of $Apc^{Min/+}$ mice.** The differential regulation of some isoforms suggested an involvement of these PKCs in the molecular processes underlying tumor formation. Because the up-regulated isoforms had recently been characterized in other animal models for intestinal cancer (20, 21), we here focused first on the two down-regulated species: *PKCα* and *PKCζ*. Both transcripts have been reported to be consistently down-regulated in other cancer

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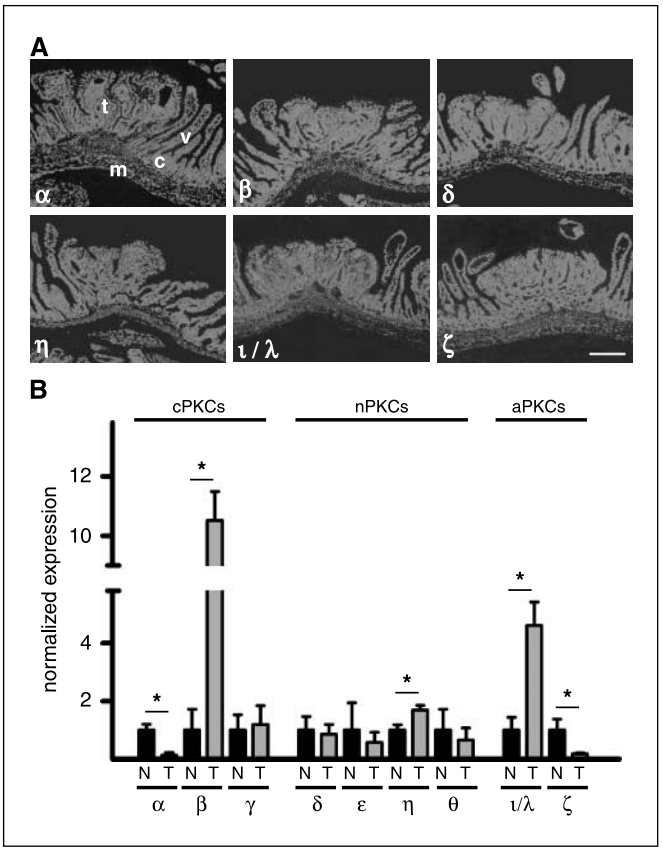

Figure 1. *PKC* mRNA expression in intestinal tumors of $Apc^{Min/+}$ mice. *A,* overlay of ISH dark field and fluorescence micrographs of intestinal tumor sections from 3-month-old $Apc^{Min/+}$ mice. *PKC* isoform–specific antisense probes used for hybridization are indicated by Greek letters. *Red,* hybridization signal; *blue,* Hoechst dye nuclear stain (*c,* crypt; *m,* muscular layers; *t,* tumor; *v,* villus). Bar, 100 μm. *B,* quantitative PCR analysis of *PKC* expression in intestinal tumor (*T*) and adjacent normal tissue (*N*). Expression values were normalized to the average normal tissue expression for each gene. *Columns,* averages (n = 3); *bars,* SE. *, $P < 0.05$, Student's t test.

models (22, 23) and in human patients (24, 25), which reassured us that the chosen mouse model is an appropriate tool for studying the role of these enzymes in human colorectal cancer.

We crossbred *PKCα*- and *PKCζ*-deficient animals with $Apc^{Min/+}$ mice to generate double-heterozygous offspring. Intercrosses from the F1 generation produced both $PKC^{+/+}$- and *PKC*-deficient $Apc^{Min/+}$ animals, which were used for further analysis. We scored intestinal polyps at three different stages. Although at 2 months of age neither PKCα- nor PKCζ-deficient animals displayed different numbers in intestinal polyps, in older animals, PKCα deletion resulted in 1.5- to 2-fold higher numbers of intestinal tumors (Fig. 2*A* and Fig. 4*B*), whereas PKCζ deletion did not have any significant effect (Fig. 2*B*).

It had previously been reported that genetic background may strongly influence malignancy in the $Apc^{Min/+}$ mouse model (26). We therefore backcrossed $Apc^{Min/+}$ $PKC\alpha^{+/-}$ mice to C57BL/6J for four generations (N4) and reevaluated tumor formation in the offspring from these animals. Tumor numbers were still increased in the absence of PKCα in these animals, confirming our observations from the mixed background (Supplementary Fig. S1*A*).

Distribution of tumors in $Apc^{Min/+}$ mice is unaffected by *PKCα* or *PKCζ* deletion. We further asked whether the frequency of tumors along the intestine has changed among the various genotypes. Therefore, we compared the distribution of lesions along the small and large intestine, and for all stages, we did not find any alterations after deletion of either *PKCα* (Fig. 2*C*) or *PKCζ* (Fig. 2*D*). Comparable results were obtained from the C57BL/6J backcrosses (Supplementary Fig. S1*B*). We therefore conclude that in the $Apc^{Min/+}$ model, PKCα suppresses tumor formation in all areas of the intestinal tract (including the colon), whereas *PKCζ* deficiency has no consequences on cancer development.

***PKCα* but not *PKCζ* deficiency reduces survival rates of $Apc^{Min/+}$ animals.** To test if the observed altered tumor formation had consequences on latency, we monitored survival in both *PKCα*- and *PKCζ*-deficient $Apc^{Min/+}$ mice. Because food composition can strongly influence intestinal tumor formation in men and mice (reviewed in ref. 27), for this study, all animals received a standardized elevated fat/protein diet after weaning. The mice were monitored daily and sacrificed when moribund. The primary cause of morbidity in all genotypes was intestinal obstruction and rectal prolapse as a result of tumors of the distal colonic and rectal region. As expected from the elevated tumor numbers, *PKCα* deficiency significantly reduced survival in $Apc^{Min/+}$ mice (Fig. 3*A*). Because *PKCζ* deletion, again, did not influence results in this setup (Fig. 3*B*), we restricted subsequent experiments on *PKCα*.

Tumors from *PKCα*-deficient $Apc^{Min/+}$ mice show a more aggressive histopathologic phenotype. Tumors from $Apc^{Min/+}$ and $Apc^{Min/+}$ $PKC\alpha^{-/-}$ littermates (ages 4 months, n = 8) were evaluated for histopathologic features. Whole preparations of full intestinal tracts revealed increased numbers of tumors penetrating the outer muscular layers of the small intestine in $PKC\alpha^{-/-}$ animals [Fig. 4*A*, *black arrows*; 7.4 ± 1.5 ($Apc^{Min/+}$ $PKC\alpha^{-/-}$) versus 2.9 ± 0.8 ($Apc^{Min/+}$ $PKC\alpha^{+/+}$) per animal]. Longitudinal sections through the small intestine and colon displayed higher numbers of tumors along the whole intestine but no significant increase in tumor diameter (Fig. 4*B*). The majority of all tumors from both genotypes were classified (according to ref. 28) as adenomas with restriction of the malignant tissue to the epithelial layers (Fig. 4*C*) and well-structured glandular architecture (Fig. 4*C, inset*). An

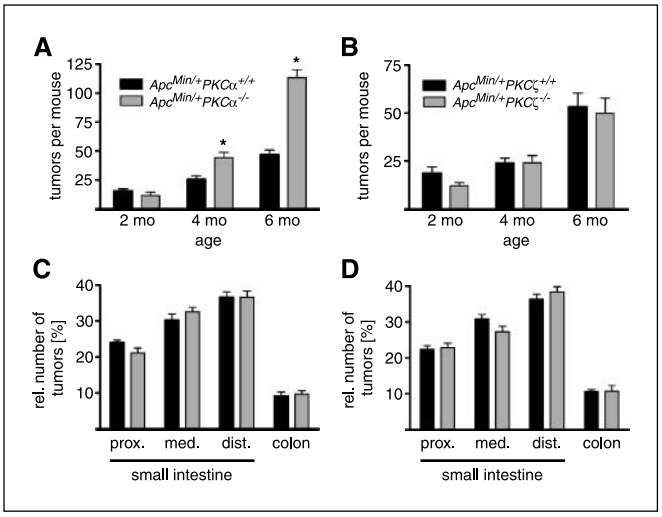

Figure 2. Intestinal tumor numbers and distribution in $Apc^{Min/+}$ and *PKCα/ζ*-deficient $Apc^{Min/+}$ mice. *A* and *B,* intestinal tumor counts for *PKCα*-deficient (*A*) and *PKCζ*-deficient (*B*) $Apc^{Min/+}$ mice and $Apc^{Min/+}$ controls at 2, 4, and 6 months of age. *C* and *D,* tumor distribution along the small intestine and the colon of *PKCα*-deficient (*A*) and *PKCζ*-deficient (*B*) $Apc^{Min/+}$ mice (ages 2-6 months). *Columns,* averages [n = 10 (*A* and *B*) or n = 30 (*C* and *D*)]; *bars,* SE. *, $P < 0.05$, Student's t test.

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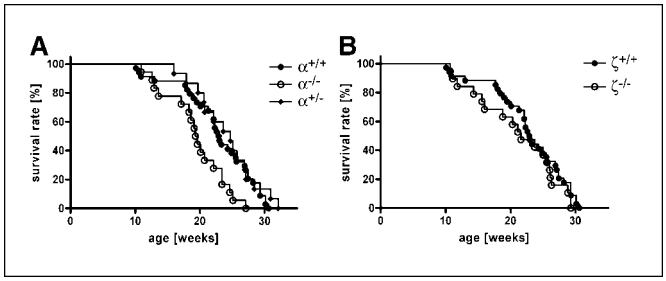

Figure 3. Survival rates of *PKCα*- or *PKCζ*-deficient *Apc*$^{Min/+}$ mice. *A,* loss of *PKCα* decreases survival rates in *Apc*$^{Min/+}$ mice [log-rank test, $P = 0.0068$; $\alpha^{+/+}$, *Apc*$^{Min/+}$ *PKCα*$^{+/+}$ ($n = 34$); $\alpha^{-/-}$, *Apc*$^{Min/+}$ *PKCα*$^{-/-}$ ($n = 18$); $\alpha^{+/-}$, *Apc*$^{Min/+}$ *PKCα*$^{+/-}$ ($n = 15$)]. *B,* deletion of *PKCζ* does not affect survival rates in *Apc*$^{Min/+}$ mice [log-rank test: $P = 0.3141$; $\zeta^{+/+}$, *Apc*$^{Min/+}$ *PKCζ*$^{+/+}$ ($n = 34$); $\zeta^{-/-}$, *Apc*$^{Min/+}$ *PKCζ*$^{-/-}$ ($n = 19$)].

elevated number of tumors in the *PKCα*-deficient animals, however, displayed more aggressive features with progression of the tumor tissue into the muscular layers, necrotic areas in central parts of the lesion (Fig. 4*D*), and overall less differentiated structure with pronounced scirrhous features (Fig. 4*D, inset*).

Taken together, these data clearly show a more aggressive nature of tumors in the *PKCα*-deficient background and may likely explain the increased mortality in these animals.

PKCα inhibits polyp formation in *Apc*$^{+/+}$ animals. The presented results thus far show a prominent role for PKCα in APC-mediated carcinogenesis. We next evaluated if a deletion of *PKCα* alone can affect tumor development in the intestinal tract. 129Sv wild-type mice do not display elevated spontaneous tumor formation (29). *PKCα*$^{-/-}$ mice are fertile and seem overall normal but show mild defects in insulin signaling and an impairment in a certain context of cerebellar long-term synaptic depression (15, 30). They do, however, not display overt predisposition to cancer during their normal lifetime (our own observations). We therefore analyzed *PKCα*$^{-/-}$ animals and age-matched wild-type controls (12 months) for small intestinal polyps and tumors. Interestingly, both wild-type and mutant animals were found to spontaneously develop sporadic lesions. The majority of these lesions were classified as aberrant crypt foci or unicryptal polyps, but sporadic adenomas of an early stage were also detected in *PKCα* deficient animals (Supplementary Fig. S2). In both genotypes, lesions were found to originate either from the epithelial cells or from the villus stroma. The muscular layer was never affected nor did any of the tumors show progression to the carcinoma stage. Overall, the number of lesions was approximately doubled in *PKCα*$^{-/-}$ mice with slightly pronounced effectiveness in the proximal parts of the intestinal tract (Fig. 5*A*), indicating that PKCα shows tumor-suppressive effects independent of the *Min* mutation. The elevated number of tumors in the *PKCα*-deficient animals was accompanied by a higher mitotic index in the intestinal crypts (Supplementary Fig. S3), indicating that PKCα suppresses tumor formation via negative regulation of cell division.

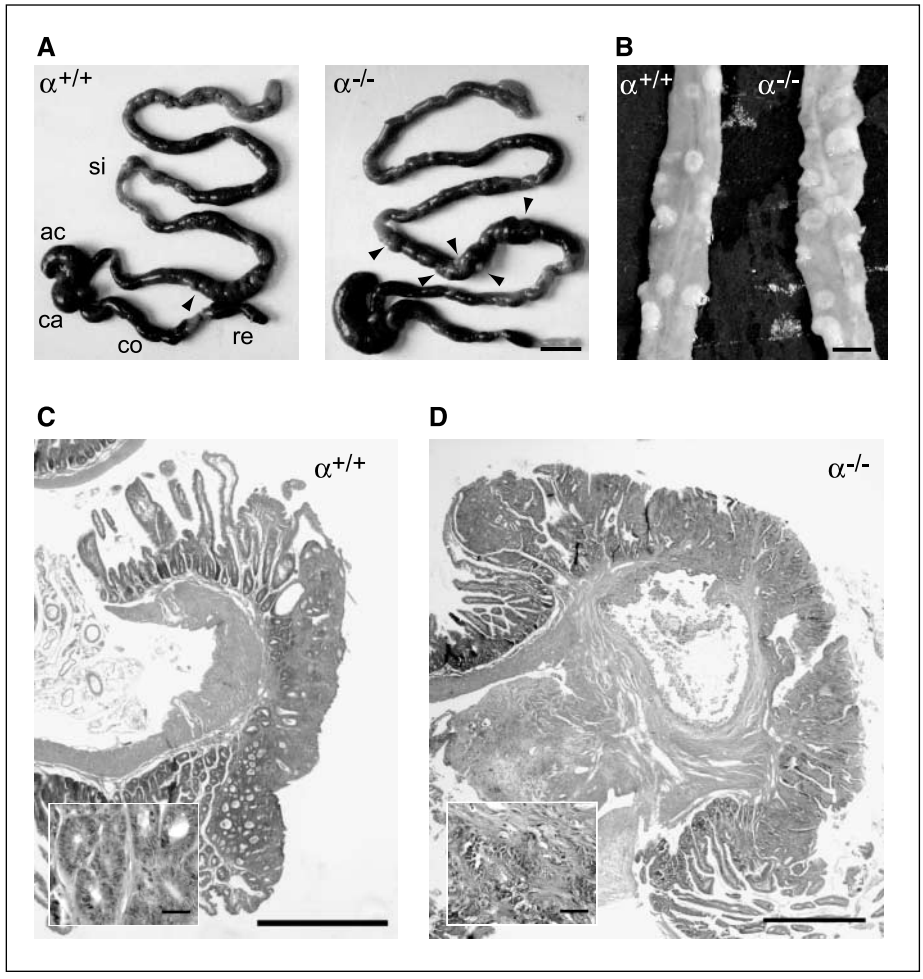

Figure 4. Deletion of *PKCα* increases tumor aggressiveness in *Apc*$^{Min/+}$ mice. *A,* whole intestine preparations of *Apc*$^{Min/+}$ *PKCα*$^{+/+}$ (*left*) and *Apc*$^{Min/+}$ *PKCα*$^{-/-}$ animals (*right*; age 3 months). *PKCα*-deficient animals show higher numbers of lesions penetrating the muscular layers (*arrowheads*). Bar, 1 cm. *B,* representative *ileum* sections from 3-month-old *Apc*$^{Min/+}$ *PKCα*$^{+/+}$ (*left*) and *Apc*$^{Min/+}$ *PKCα*$^{-/-}$ animals (*right*). *PKCα* deficiency causes a 1.5- to 2-fold increase in tumor numbers along the whole intestinal tract. Bar, 5 mm. *C* and *D,* micrographs of H&E-stained tumor sections. *C,* papillary adenoma from an *Apc*$^{Min/+}$ animal without penetration of the muscular layers. Bar, 1 mm. *Inset,* higher magnification of the same tumor shows a well-organized glandular structure of the tumor tissue. Bar, 50 μm. Adenocarcinoma from an *Apc*$^{Min/+}$ *PKCα*$^{-/-}$ animal with ulcerated core and penetration into the muscular layers. Bar, 1 mm. *Inset,* higher magnification of the same tumor, depicting reduced glandular and enhanced scirrhous properties of the tumor tissue. Bar, 50 μm.

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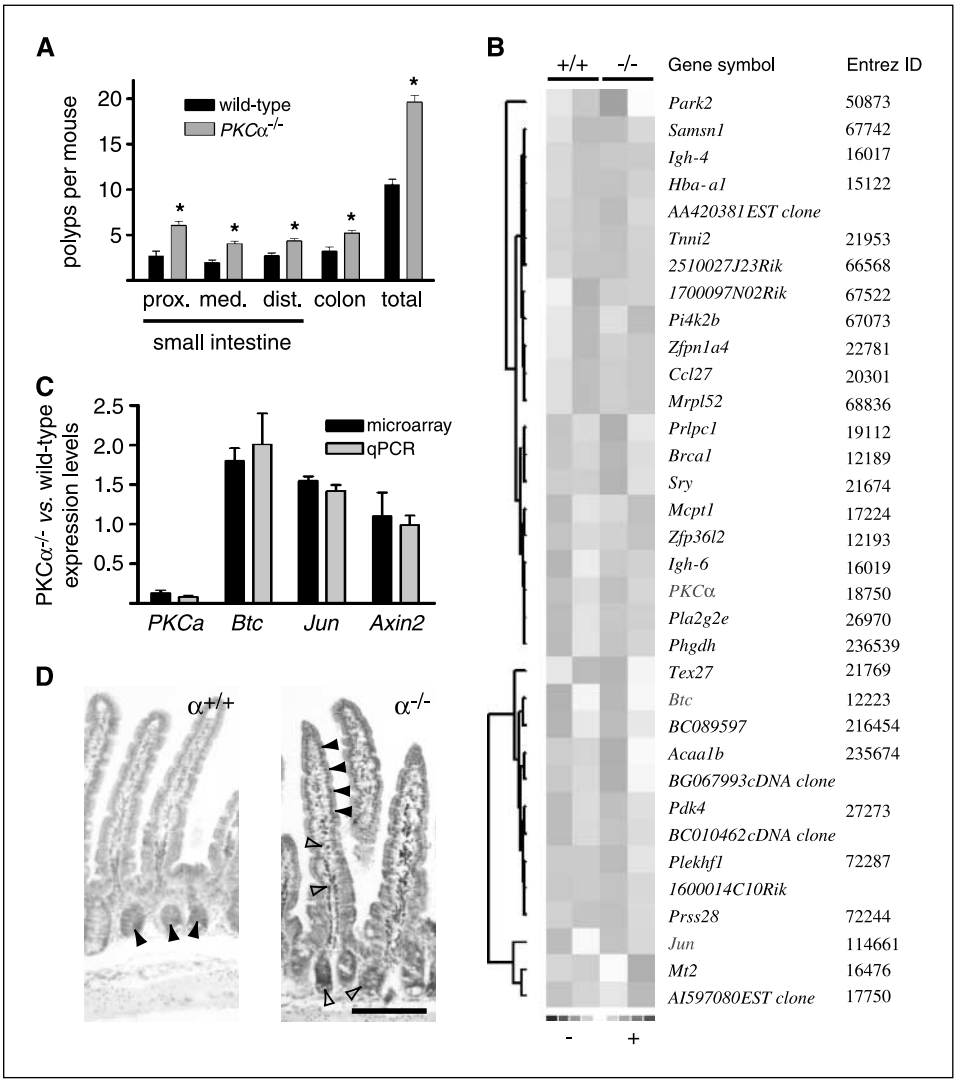

Figure 5. Polyp formation and transcriptional profiling in the *PKCα*-deficient intestine. *A,* intestinal polyp counts in 12-month-old *PKCα*$^{-/-}$ animals and congenic wild-type controls. *Columns,* mean (n = 8); *bars,* SE. *, $P < 0.05$, Student's t test. *B,* microarray expression analysis from *PKCα*$^{-/-}$ and wild-type small intestine preparations. Hierarchical clustering of transcripts found to be up-regulated or down-regulated >1.5-fold (+/+, wild-type samples; −/−, *PKCα*$^{-/-}$ RNA preparations; *blue,* low expression; *red,* high expression). Probe sets in red were used for validation. *C,* validation of microarray results by quantitative PCR. Mutant versus wild-type expression values for *PKCα, Btc, Jun*, and the WNT target *Axin2*. *Columns,* averages [n = 2 (microarray) and n = 3 (quantitative PCR)]; *bars,* SE. *D,* BTC protein expression in the wild-type (*left*) and *PKCα*-deficient intestine (*right*). Bar, 100 μm.

Transcriptional profiling in the *PKCα*$^{-/-}$ intestine reveals up-regulation of *Btc* and its target *Jun* but shows no effect on WNT target gene expression. To identify the molecular targets conferring the tumor-suppressive effects of PKCα in the murine intestine, we did transcriptional profiling by oligonucleotide microarray analysis on small intestine preparations from wild-type and PKCα-deficient animals. After normalization, we found a relatively small number of significantly regulated genes, the strongest of which (≥1.5-fold up-regulated or down-regulated) are listed in Fig. 5*B*. *PKCα* was strongly down-regulated (7.7-fold) as would be expected in the *knockout*. Interestingly, among the up-regulated genes, we found two known oncogenes: the EGF *Btc* (31) and the activating protein-1 (AP-1) component *Jun* (reviewed in ref. 32). The microarray results were evaluated by quantitative PCR on a couple of selected genes (Fig. 5*C*). As expected, *PKCα* was found to be absent in the *PKCα*$^{-/-}$ intestine in both set ups (7.7-fold versus 12.5-fold down-regulation, microarray versus quantitative PCR, respectively), whereas *Btc* and *Jun* seemed consistently up-regulated (1.8-fold versus 2.0-fold and 1.5-fold versus 1.4-fold, respectively). The up-regulation of *Btc* was confirmed on the protein level. Although in the wild-type intestine BTC was only detected in the Paneth cells (Fig. 5*D, black arrows*), strong expression was seen in epithelial and stroma cells of the *PKCα*-deficient intestine (Fig. 5*D*, *black* and *open arrows*). Expression of WNT target genes, like *Axin2*, was not significantly different between the wild-type and *PKCα*$^{-/-}$ intestine (Fig. 5*C*).

Taken together, these data further strengthen our previous finding that PKCα deficiency does not directly affect WNT/APC signaling. They instead propose a role for this kinase in the regulation of the EGFR/AP-1 pathway.

PKCα and APC influence EGF signaling via synergistic regulation of EGFR target gene transcription. To further corroborate these findings, we evaluated transcriptional regulation of WNT/APC and EGFR/AP-1 downstream target genes in the different *Apc* and *PKCα* genotypes. In agreement with other reports, the WNT target *Axin2* was up-regulated in the *Apc*$^{Min/+}$ normal intestine with even higher expression in tumor tissues (Fig. 6*A*). An additional deletion of *PKCα*, however, did not influence this transcriptional activation (Fig. 6*A*). On the other hand, *Btc*, which is itself AP-1 regulated, was not significantly up-regulated by the *Apc* mutation alone. However, a deletion of PKCα increased *Btc* transcript levels, and this effect was potentiated in the *Apc*$^{Min/+}$ *PKCα*$^{-/-}$ intestine (Fig. 6*B*). Similar regulation was observed for the AP-1 target *Jun* (data not shown). We therefore

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speculated that due to the up-regulation of an EGFR ligand in the PKCα deficiency, EGFR signaling via an autocrine loop might be increased. EGFR activation is accompanied by autophosphorylation at several tyrosine residues. We assessed EGFR activation by phospho-specific antibodies in the *PKCα*-deficient intestine. Deletion of *PKCα* increased EGFR phosphorylation in both wild-type and $Apc^{+/min}$ animals (Fig. 6*C*), indicating that the observed transcriptional response is indeed mediated via enhanced EGFR signaling.

A common mediator of EGFR signaling is the mitogen-activated protein kinase (MAPK) signaling cascade. We checked for the activation of this pathway by immunohistochemically detecting the activated (phosphorylated) form of ERK1 and ERK2 in *Apc* and *PKCα* mutant tissues (Fig. 6*D*). Although in the wild-type intestine activated ERK1/2 was exclusively detected in Paneth cells and the bottom of the crypts, in the *PKCα* mutant, we in addition found elevated phospho-ERK levels all along the rim of the villi. In $Apc^{Min/+}$ animals, ERK phosphorylation was restricted to Paneth cells and crypts but extended significantly more from the bottom of the crypts towards the tip of the villi. In $Apc^{Min/+}$ PKCα$^{-/-}$ mice, however, phospho-ERK immunoreactivity was strongly detected in the Paneth cells as well as all along the border of the intestinal mucosa. This increased ERK1/2 activation was not paralleled by an elevated protein level of ERK1/2; instead, ERK1/2 base levels were comparable in all genotypes (Supplementary Fig. S4).

In summary, these results suggest a role of PKCα in the regulation of EGFR signaling via inhibition of the MAPK pathway. In the case of an *Apc* mutation, EGFR and WNT signaling converge on the transcription of AP-1 target genes and by that may synergistically promote intestinal tumor formation.

Discussion

In this study, we have shown a role for PKCα as tumor suppressor in intestinal cancer. Deletion of *PKCα* but not of *PKCζ* promotes polyp formation in wild-type mice and in the $Apc^{Min/+}$ tumor model. In the latter, activation of EGFR (via *PKCα* deletion) and WNT (via APC mutation) pathways synergistically induce AP-1 target genes, resulting in increased numbers of tumors and accelerated progression to more aggressive stages.

We started this analysis by evaluating *PKC* transcription in normal tissue and in early tumors of the $Apc^{Min/+}$ intestine. Previous studies had already been published summarizing PKC expression in this mouse model based on antibody (33) or

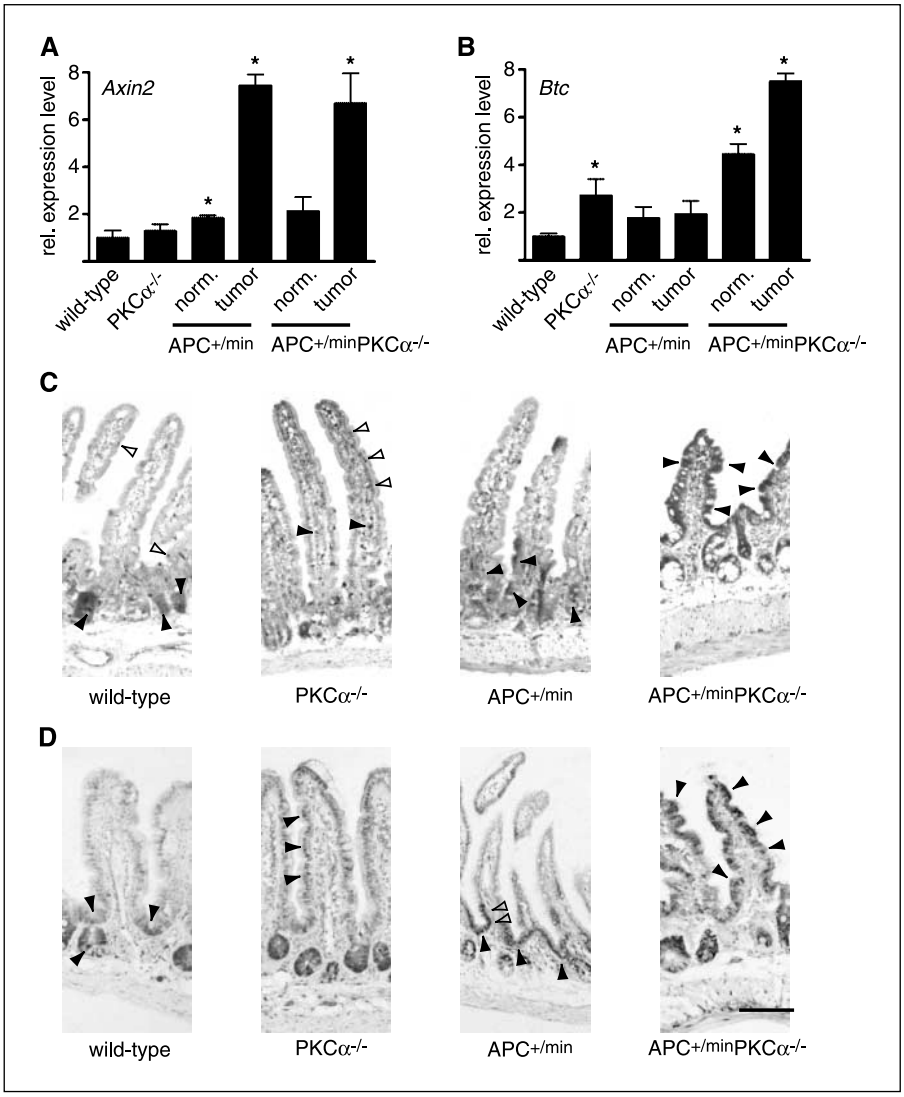

Figure 6. Synergistic effects of APC and PKCα on WNT and EGFR signaling. *A,* APC/β-catenin/TCF–mediated activation of the WNT target gene *Axin2* in $Apc^{Min/+}$ mice is unaffected by a deletion of *PKCα* as determined by quantitative PCR analysis of tumor and normal intestine preparations. *B,* the EGRF/AP-1 target gene *Btc* is synergistically activated by the APC mutation and the deletion of *PKCα* (quantitative PCR analysis). *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$, Student's *t* test. *C* and *D,* immunohistochemical detection of activated EGFR (*C*) and ERK1/2 (*D*) in wild-type, PKCα$^{-/-}$, $Apc^{Min/+}$, and $Apc^{Min/+}$ PKCα$^{-/-}$ intestinal sections. In the wild-type intestine, activation of EGFR was restricted to the bottom of the crypts (*black arrowheads* in *C*) and few cells in the villus epithelium (*open arrowheads*). *PKCα* deficiency resulted in increased numbers of phospho-EGFR–positive cells in both epithelial (*open arrowheads*) and stromal cells (*black arrowheads*). $Apc^{+/min}$ animals showed increased phospho-EGFR in the proliferative zone (*black arrowheads*) but no difference in the outer villi when compared with wild-type animals. In $Apc^{Min/+}$ PKCα$^{-/-}$ animals, strong EGFR activation was observed along the whole intestinal epithelium. In wild-type animals, phospho-ERK signal was restricted to the proliferative crypts and the Paneth cells (*arrowheads* in *D*). In the *PKCα*-deficient intestine, elevated ERK1/2 phosphorylation was also detected along the villus epithelium (*arrowheads*). $Apc^{Min/+}$ animals show increased staining in the crypts (*black arrowheads*), progressing along the inner villus epithelium (*open arrowheads*), whereas in $Apc^{Min/+}$ PKCα$^{-/-}$ animals, phospho-ERK signal can be found all over the intestinal epithelium (*arrowheads*). Activated EGFR was detected with an antibody directed against the phosphorylated receptor (pY-1068). Bar, 100 μm.

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microarray approaches (34, 35), in some cases with quite contrary results. For example, *PKCβ* was found to be up-regulated in $Apc^{Min/+}$ adenomas by Leclerc et al., weakly down-regulated by Klein et al., and both up-regulated and down-regulated (depending on the probe set) at the same time by Paoni et al. Both microarray (sample dissection and processing) and immunohistochemistry (antibody specificity) set ups have their pitfalls; thus, we chose RNA ISH as our main approach. All probes were carefully checked for sequence homologies to guarantee isotype specificity, whereas the use of ^{35}S labeling allows for a faithful estimation of expression levels with a resolution close to that of antibody stainings. In addition, quantitative PCR was used to validate the results obtained from the ISH data. Overall, our data mostly confirmed those from the abovementioned studies. Specifically, *PKCα* down-regulation was reported by both Paoni and Klein, whereas no clear regulation was seen by Leclerc. The results for *PKCβ* were quite contrary in the previous reports (see above), but patient data (e.g., ref. 36) and reports from other animal models (37, 38) support our and Leclerc's findings. Weak regulation of *PKCδ* was also seen in all other studies. Our *PKCη* data fits to those from Klein, but opposing regulation was reported from Paoni et al. These different findings likely result from the comparably low overall expression of this enzyme. Interestingly, PKCη has been implicated in mediating cancer development and wound healing in the skin, and *PKCη* expression is elevated in proliferating epidermal cells during wound closure (39). Our data about up-regulation of *PKCι/λ* in $Apc^{Min/+}$ tumors are not confirmed by Paoni and Leclerc (whereas PKCι/λ was not tested by Klein et al.), but they support results obtained from patients and other animal models (21). For *PKCζ*, the previous studies (with the exception of Leclerc) agree with us on a down-regulation of this gene in tumor tissue, which again is consistent with patient data (40) and other animal models (37).

The down-regulation of *PKCα* and *PKCζ*, the two isoforms selected for further studies, suggested a potential role of these kinases in the molecular events that accompany cell transformation. For PKCα numerous, mostly cell-based reports have been published attributing both tumor-promotive and tumor-suppressive properties to this kinase in colorectal cancer (reviewed in ref. 12). Despite the fact that PKCα could possibly act as a tumor suppressor, two clinical studies using PKCα antisense inhibitors have already been done in human cancer patients (41, 42), without conclusive results. Literature on PKCζ in intestinal cancer is still sparse (most likely due to lack of specific antibodies or pharmacologic tools to characterize this enzyme), but PKCζ seems to be involved in polyp progression from intestinal myofibroblasts (43) and may control β-Catenin levels via regulation of GSK-3β activity (44). To analyze the involvement of these two kinases in intestinal cancer development *in vivo*, we therefore chose the $Apc^{Min/+}$ mouse model in combination with our established *knockout* models of PKCα and PKCζ.

In summary, our data suggest that PKCζ does not play a role in tumorigenesis in this model. However, the high structural homology and overlapping substrate specificity between PKCζ and the other atypical PKC isoform (45) offer the possibility that PKCι/λ may in the absence of PKCζ take over some of its functions and thereby mask the PKCζ-deficient phenotype.

The function of PKCα, on the other hand, seems not to be redundant with other PKCs in this set up because the loss of this enzyme markedly increased tumor numbers at different stages and reduced survival rates in $Apc^{Min/+}$ mice. This effect was not restricted to certain parts of the intestine but was observed throughout the small intestine and the colorectal track. The increased morbidity in these animals is most likely a result of the more aggressive morphology of the *PKCα*-deficient tumors. Excessive growth of adenocarcinomas into the peritoneal lumen increases external pressure on the intestinal walls and may thereby affect gut motility and raise the risk of obstructions, the primary cause of morbidity in $Apc^{Min/+}$ animals.

The accelerated tumor progression observed in *PKCα*-deficient mice suggests that in these animals, tumor metastasation may be increased as well. In concordance with previous reports (46), we sporadically detected secondary tumors in mammary glands of both genotypes but did not observe an increased risk of metastasation in *PKCα*-deficient mice. This is most likely a consequence of the reduced life span of these animals, which may normally not suffice to allow the formation of these lesions. Crossbreedings of $Apc^{Min/+}$ $PKC\alpha^{-/-}$ animals with more resistant strains like AKR/J (26) or transplantation of tumors into nude mice could be used to answer the question of metastasation in the *PKCα*-deficient background.

Strikingly, a deletion of *PKCα* favors spontaneous polyp formation already in the absence of an APC mutation. Transcriptional profiling revealed that in the *PKCα*-deficient intestine, expression of the oncogenes *Btc* and *Jun* is increased, whereas WNT target genes, like *Axin2*, are unaffected. On the first view, this was surprising to us. Due to reports that clearly indicate a functional role for classic PKCs in the context of GSK-3β activity (47), we were expecting to see an alteration in the WNT pathway associated with a destabilization of β-CATENIN. On the other hand, PKCβ has already been identified as a regulator of GSK-3β activity and its signaling in another colon cancer model (20). Thus, it could well be that PKCβ is the classic PKC isoform that regulates, among other kinases, GSK-3β activity. Nevertheless, we cannot exclude from our data that in the wild-type situation, PKCα is also capable to modulate GSK-3β activity, but this function might be covered in the deficiency due to redundancy among the PKC family.

PKCα seems to negatively regulate proliferation in the intestinal crypts. Elevated numbers of dividing cells were mostly detected in the epithelial cells of *PKCα*-deficient mice. However, some of the early-stage lesions detected in the intestinal tract of these animals were originating from the villus stroma. Although the restriction of elevated ERK and EGFR signaling to epithelial cells in the *Apc/PKCα* double mutant animals indicates that *PKCα* primarily influences carcinogenesis in this tissue, further studies are needed to clarify its role in other cell types of the intestinal tract.

The EGF family member *Btc* and *Jun* are established oncogenes acting via stimulation of cell growth and proliferation (48, 49). *Btc* was found to be moderately expressed in the intestinal epithelium (50); injection of a synthetic peptide may increase the depth of colonic crypts (51); and its elevated expression is a negative prognostic marker for rectal cancer (52). BTC is able to bind and activate several avian erythroblastic leukemia viral oncogene homologue (ERBB) type receptors (53), thereby signals via the MAPK cascade (49, 54). JUN is one component of the AP-1 transcriptional activator complex, an important regulator of cell proliferation (55). Strikingly, AP-1 activation is a major target of BTC and ERBB-mediated signal transduction (56). In concordance with this, we detected activation of EGFR after deletion of *PKCα* in both $Apc^{+/+}$ and $Apc^{Min/+}$ animals. Important factors in these signaling processes are ERK kinases (57), which we found more

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ubiquitously activated in the intestinal mucosa in the absence of PKCα. The observed negative regulation of ERK1/2 activation by PKCα that we report here is in marked contrast to the ERK-mediated positive influence of this enzyme on apoptosis in the CaCo2 colon tumor cell line (58). This could be attributed to a cell type or *species* effect, or it may reflect differential functionality of PKCα and ERK signaling during different developmental stages of intestinal epithelial and tumor cells. On the other hand, PKCα has specifically been shown to inhibit ERK signaling in lung fibroblasts (59), in HeLa cells downstream of hepatocyte growth factor/c-MET signaling (60), and after cytochalasin D treatment of embryonal mesenchymal cells (61).

We provide evidence that in the $Apc^{Min/+}$ mouse model, *PKCα* deficiency–mediated signaling and the WNT pathway synergistically interact in promoting tumor formation and progression despite a direct role of PKCα in APC signal transduction. A likely explanation may be the convergence of both WNT and EGFR pathways on the transcriptional activation on AP-1 target genes like found for *Jun* and *Btc*. In this context, recent work by Nateri et al. (62) has shown that WNT-activated TCF-4 and JUN directly interact with β-CATENIN to activate AP-1 target genes, thus regulating intestinal cancer development *in vivo*.

PKCα may interfere directly with EGFR signaling via inhibitory pathways. Early work has shown that cPKCs can themselves phosphorylate EGFR at Thr^{654}, thereby decreasing the ligand affinity and signaling capacity of this receptor (63–65). Additionally, in a recent report, PKCα has been shown to phosphorylate protein tyrosine phosphatase PTP-1C, which in turn deactivates EGFR and decreases ERK phosphorylation in response to retinoid treatment in breast cancer cells (66). Taken together, these data support our notion that EGFR signaling is altered in the PKCα-deficient intestine.

In conclusion, we report that *PKCα* and *PKCζ* transcripts are down-regulated in intestinal tumors of $Apc^{Min/+}$ mice. Deletion of *PKCα* but not of *PKCζ* promotes tumor formation and progression and reduces survival rates in $Apc^{Min/+}$ animals. Even in wild-type animals, *PKCα* deficiency favors spontaneous polyp formation but with a much milder phenotype. Thus, we have shown that PKCα, an originally identified phorbol ester binding protein, acts as a tumor suppressor in the $Apc^{Min/+}$ model. PKCα negatively regulates expression of *Btc* and *Jun* and activation of the MAPK cascade. Together, these data suggest that EGFR/PKCα and WNT/APC signaling pathways may converge on the level of transcriptional activation of AP-1 target genes, thereby synergistically promoting intestinal tumorigenesis.

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