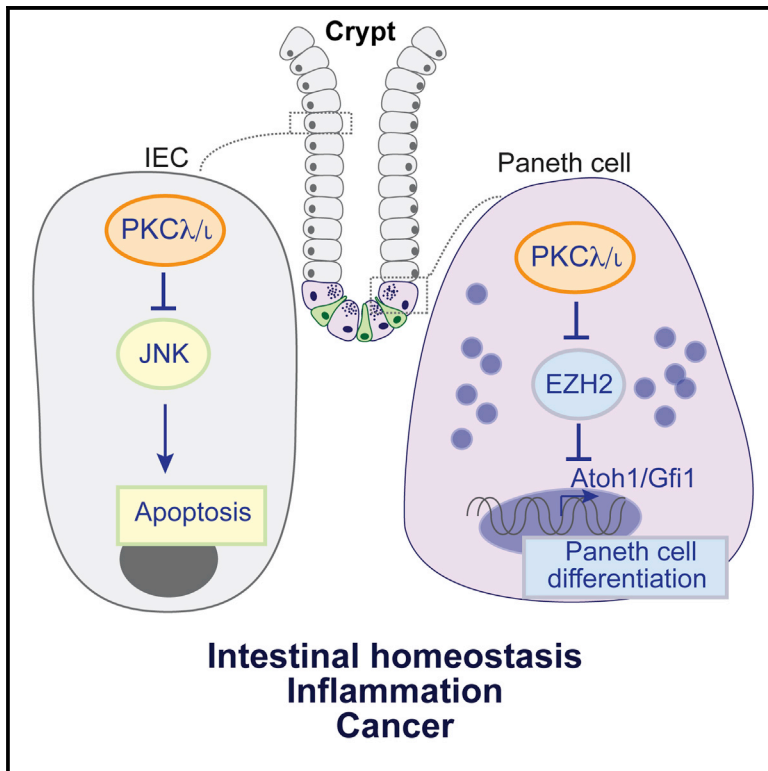


Control of Paneth Cell Fate, Intestinal Inflammation, and Tumorigenesis by PKC λ /i

Graphical Abstract



Authors

Yuki Nakanishi, Miguel Reina-Campos, Naoko Nakanishi, ..., Richard S. Blumberg, Maria T. Diaz-Meco, Jorge Moscat

Correspondence

jmoscat@sbsdsc.discovery.org

In Brief

Nakanishi et al. demonstrate that the atypical PKC λ /i is lost in the intestinal epithelium of Crohn's disease patients, and its genetic inactivation in mice results in defective Paneth cell differentiation, enhanced JNK activity, and apoptosis in the intestinal epithelium, which leads to increased intestinal inflammation and tumorigenesis.

Highlights

- PKC λ /i is required for Paneth cell differentiation
- PKC λ /i reduces EZH2 stability and promotes Atoh1 and Gfi1 expression
- PKC λ /i induces intestinal epithelial cell survival through the repression of JNK
- PKC λ /i loss enhances intestinal inflammation and cancer

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Control of Paneth Cell Fate, Intestinal Inflammation, and Tumorigenesis by PKC λ /I

Yuki Nakanishi,¹ Miguel Reina-Campos,¹ Naoko Nakanishi,¹ Victoria Llado,¹ Lisa Elmen,² Scott Peterson,² Alex Campos,³ Surya K. De,⁴ Michael Leitges,⁵ Hiroki Ikeuchi,⁶ Maurizio Pellecchia,⁴ Richard S. Blumberg,⁷ Maria T. Diaz-Meco,¹ and Jorge Moscat^{1,8,*}

¹Cancer Metabolism and Signaling Networks Program

²Bioinformatics and Structural Biology Program

³Proteomics Facility

Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

⁴Division of Biomedical Sciences, School of Medicine, University of California, Riverside, 900 University Avenue, Riverside, CA 92521, USA

⁵Biotechnology Centre of Oslo, University of Oslo, 0316 Oslo, Norway

⁶Department of Surgery, Hyogo College of Medicine, Nishinomiya 663-8131, Japan

⁷Division of Gastroenterology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

⁸Lead Contact

*Correspondence: jmoscat@sbpdiscovery.org
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SUMMARY

Paneth cells are a highly specialized population of intestinal epithelial cells located in the crypt adjacent to Lgr5⁺ stem cells, from which they differentiate through a process that requires downregulation of the Notch pathway. Their ability to store and release antimicrobial peptides protects the host from intestinal pathogens and controls intestinal inflammation. Here, we show that PKC λ /I is required for Paneth cell differentiation at the level of Atoh1 and Gfi1, through the control of EZH2 stability by direct phosphorylation. The selective inactivation of PKC λ /I in epithelial cells results in the loss of mature Paneth cells, increased apoptosis and inflammation, and enhanced tumorigenesis. Importantly, PKC λ /I expression in human Paneth cells decreases with progression of Crohn's disease. Kaplan-Meier survival analysis of colorectal cancer (CRC) patients revealed that low *PRKCI* levels correlated with significantly worse patient survival rates. Therefore, PKC λ /I is a negative regulator of intestinal inflammation and cancer through its role in Paneth cell homeostasis.

INTRODUCTION

The control of intestinal homeostasis relies on a perfectly orchestrated balance of interactions among the different cell types of the intestinal epithelium, microbiota, and immune system (Medema and Vermeulen, 2011). The gut epithelium undergoes continuous self-renewal from intestinal stem cells (ISCs) located in the proliferative crypt (van der Flier and Clevers, 2009). Paneth cells are critical to the control of the ISC niche and the intestinal barrier (Adolph et al., 2013; Clevers and Bevins, 2013). This is a highly specialized population of intestinal epithelial cells (IECs)

located adjacent to Lgr5⁺ ISC, which differentiate from ISCs upon downregulation of the Notch pathway (Fre et al., 2005; van Es et al., 2005). Paneth cells have large granules containing lysozyme and other peptides, such as defensins/cryptidins, that serve to protect the host from intestinal pathogens (Clevers and Bevins, 2013) and play a critical role in the control of intestinal inflammation (Adolph et al., 2013). Alterations in the normal function of IECs, especially Paneth cells, contribute to pathologies like inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC) (Kaser et al., 2010). Importantly, patients with IBD are at an increased risk for colorectal cancer (CRC) (Jess et al., 2006). Patients with CD often exhibit a reduced number of healthy Paneth cells and decreased expression of defensins in areas of acute inflammation (Wehkamp et al., 2008). Therefore, understanding the signaling cascades that regulate Paneth cell differentiation and function, as well as their role in the control of intestinal homeostasis and pathology, is critical for the design of new therapies for these diseases.

Here, we have addressed this biological problem in the context of the role and mechanisms of action of protein kinase C (PKC) λ /I. This kinase, along with PKC ζ , constitutes the atypical PKC family (Moscat et al., 2009). Both have been implicated in several oncogenic and inflammatory pathways in vitro, but their role in intestinal homeostasis and pathology has only recently begun to be investigated in physiologically relevant mouse models (Calcagno et al., 2011; Llado et al., 2015; Ma et al., 2013; Moscat et al., 2009). In this regard, our laboratory previously reported a specific tumor-suppressor role of PKC ζ in intestinal carcinogenesis via the inhibition of metabolic stress reprogramming (Ma et al., 2013), as well as the β -catenin and Yap pathways in ISCs (Llado et al., 2015). In contrast, PKC λ /I has been proposed by others to be a tumor promoter (Justilien et al., 2014). However, the recently reported analysis of PKC mutations identified in human cancers strongly suggested that most of these mutations led to loss of function and none would result in a gain-of-function phenotype (Antal et al., 2015). These studies

are in good agreement with our data demonstrating that PKC ζ is a tumor suppressor in several types of neoplasia, including intestinal cancer (Galvez et al., 2009; Kim et al., 2013; Ma et al., 2013), but they are at odds with the pro-tumorigenic role of PKC $\lambda/1$ proposed by others (Murray et al., 2004).

However, when considering cancer etiology and progression, cross-talk between the tumor cells and the surrounding microenvironment must be taken into account. In this regard, the etiology of intestinal cancer is complex, because disruption of the epithelial barrier, even under conditions in which inflammation is not the driver of the carcinogenic process, or as consequence of its erosion during IBD, inevitably results in the hyperproduction of inflammatory cytokines that enhance tumor progression (Grivennikov et al., 2012; Karin and Clevers, 2016). Therefore, at least in intestinal carcinogenesis, and most likely in all tumors, the existence of activating or inactivating mutations in a given gene should be put in the context of other factors driving the inflammatory and immunological landscape of the tumor. In this paper, we have established the role of PKC $\lambda/1$ in the intestinal epithelium, and especially Paneth cells, in inflammation and cancer.

RESULTS

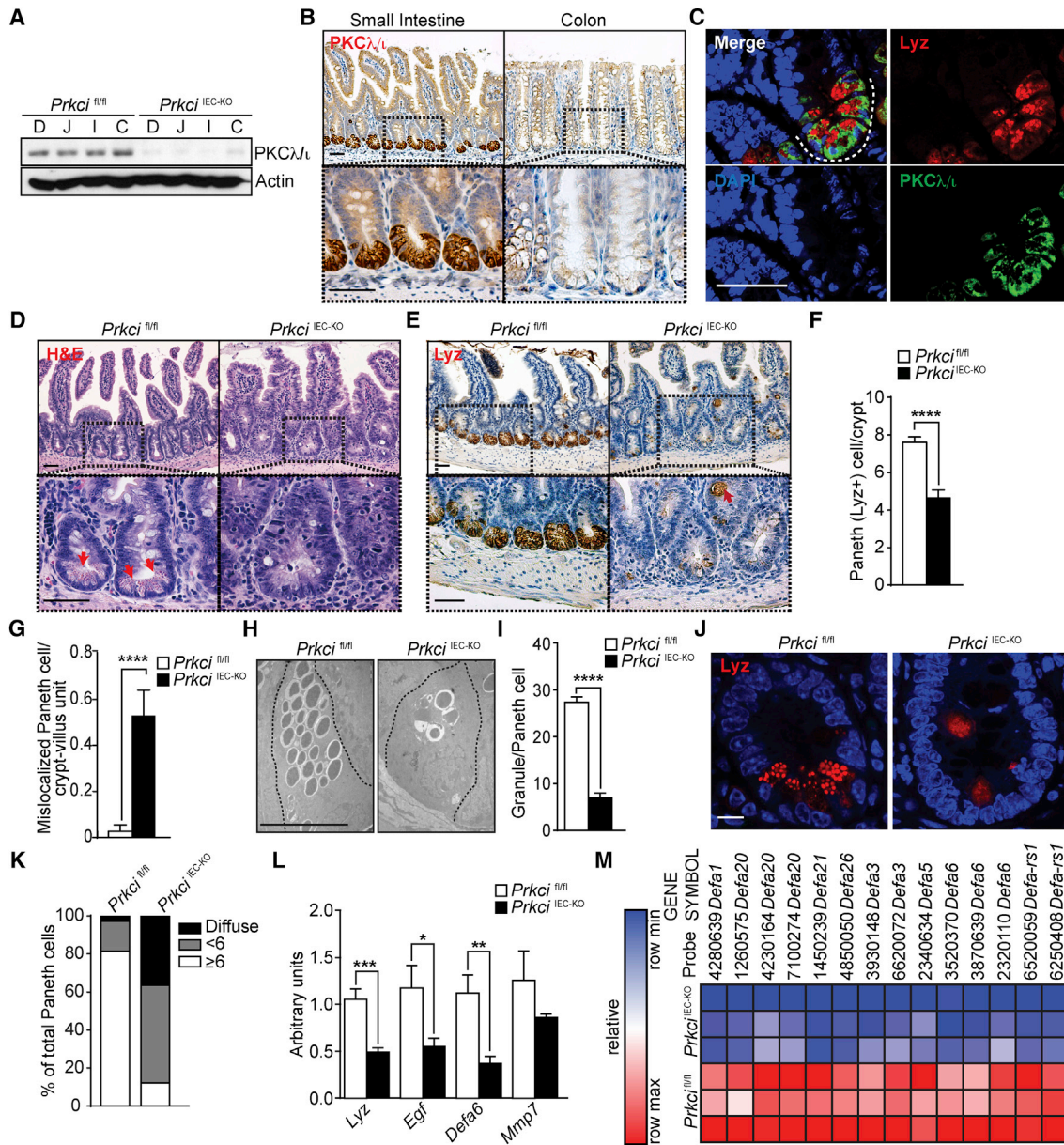
Paneth Cell Defects in PKC $\lambda/1$ -Deficient Intestinal Epithelial Cells

PKC $\lambda/1$ is widely expressed in the small intestine and colon, as determined by immunoblot analysis of IECs (Figure 1A), which suggested a role for PKC $\lambda/1$ in regulating homeostasis in the gastrointestinal tract. Although all IECs of the small intestine and colon expressed PKC $\lambda/1$, its levels were much higher in Paneth cells at the crypt base (Figures 1B and S1A). This was confirmed by double immunofluorescence (IF) analysis of intestinal crypts in which we found an almost complete colocalization of PKC $\lambda/1$ with lysozyme (Lyz), a well-established marker of Paneth cells (Figures 1C and S1B). To investigate the potential role of PKC $\lambda/1$ in intestinal homeostasis, we crossed *Prkci*^{fl/fl} mice with Villin-cre mice to generate a mouse line lacking PKC $\lambda/1$ specifically in IECs (*Prkci*^{IEC-KO} mice). *Prkci*^{IEC-KO} mice were born at the expected Mendelian ratios and developed normally. Epithelial PKC $\lambda/1$ deficiency had no effect on the overall structures of the ileum or the colon, and it is unlikely that this was due to compensation, because expression of other PKCs was not altered in *Prkci*^{IEC-KO} crypts (Figure S1C). However, we detected enlargement of the crypts and a severe reduction in the number of Paneth cells, as identified by the absence of granule-containing cells in H&E-stained sections and a strong reduction in Lyz staining in the mutant mice (Figures 1D–1F). This was also accompanied by the presence of mislocalized Lyz⁺ cells in the upper crypt and villi of *Prkci*^{IEC-KO} intestines (Figures 1E and 1G). Staining of markers of other differentiated cell populations did not show alterations in *Prkci*^{IEC-KO} mice (Figure S1D). Electron microscopy (EM) analysis also showed loss of mature Paneth cells in *Prkci*^{IEC-KO} crypts and revealed alterations in the morphology of the remaining Paneth cells, which displayed reduced numbers of granules (Figures 1H and 1I). Further analysis by Lyz staining showed that in contrast to normal Paneth cells in which Lyz is packaged efficiently in the granules, in *Prkci*^{IEC-KO} mice, there was an increase in Paneth

cells with reduced numbers of Lyz-containing granules and with diffuse Lyz staining (Figures 1J and 1K). Consistently, *Prkci*^{IEC-KO} crypts showed decreased expression of Paneth cell-related gene transcripts (Figure 1L). Furthermore, genome-wide transcriptomic analysis identified downregulation of defensin transcripts in the PKC $\lambda/1$ -deficient crypts (Figures 1M). These results suggest that PKC $\lambda/1$ deficiency in IECs caused a disruption of Paneth cell homeostasis.

PKC $\lambda/1$ Deficiency Increases Intestinal Epithelial Cell Death

Histological analysis of *Prkci*^{IEC-KO} intestines showed a large number of dying cells with pyknotic nuclei at the crypt and increased numbers of apoptotic cells positive for cleaved caspase-3 and TUNEL, as compared to *Prkci*^{fl/fl} intestines, in which apoptotic cells were mostly detected only at the villus tip (Figures 2A–2D, S2A, and S2B). EM sections also revealed increased numbers of dead cells, including necrotic cells, in *Prkci*^{IEC-KO} intestines, accompanied by infiltration of macrophages that engulfed dead cells (Figures 2E and 2F). Double IF of Lyz and TUNEL showed that most of the few remaining Paneth cells in the crypts of *Prkci*^{IEC-KO} mice were TUNEL positive (Figures 2G and S2C), suggesting that immature PKC $\lambda/1$ -deficient Paneth cells also underwent cell death under basal conditions. Crypt apoptosis was also detected in the colon of *Prkci*^{IEC-KO} mice (Figure S2D). NextBio analysis of the upregulated genes in the *Prkci*^{IEC-KO} crypts showed a strong correlation with the Gene Ontology (GO) category “positive regulation of programmed cell death” (Figure 2H). These results demonstrate that PKC $\lambda/1$ loss not only results in defective Paneth cells but also sensitizes crypt cells to apoptosis. Therefore, we next focused our attention on the molecular mechanisms whereby PKC $\lambda/1$ regulates crypt cell survival. Interestingly, gene set enrichment analysis (GSEA) identified “AP1 pathway” as a significantly upregulated category in *Prkci*^{IEC-KO} crypts (Figure 2I). JNK activation is a major component of the AP1-driven cell death cascades (Davis, 2000). Interestingly, the staining of p-JNK and p-c-Jun was dramatically increased in the crypts of *Prkci*^{IEC-KO} mice as compared to *Prkci*^{fl/fl} mice (Figure 2J). Immunoblot analysis of crypts confirmed increased JNK and caspase-3 activity associated with PKC $\lambda/1$ deficiency (Figure 2K). Since JNK is activated by tumor necrosis factor (TNF), we hypothesized that PKC $\lambda/1$ deletion would sensitize IECs to TNF, which would further increase JNK activation and cell death, thus creating an amplifying loop resulting in increased inflammation. In keeping with this concept, when crypt organoid cultures from *Prkci*^{IEC-KO} mice were treated with TNF or interferon- γ (IFN- γ), two cytokines highly abundant in the intestinal inflammatory milieu, the result was enhanced JNK activation and concomitant elevation of cleaved caspase-3 (Figure 2L). To test whether this was a cell-autonomous effect, we used SW480 as a model system of human intestinal epithelial cells. Basal JNK activation was highly upregulated in SW480 cells in which PKC $\lambda/1$ was knocked down by small hairpin RNA (shRNA), which was further enhanced by TNF (Figure 2M). To establish the role of JNK activation in apoptosis triggered by PKC $\lambda/1$ deficiency, PKC $\lambda/1$ -knockdown cells were treated with a cell-permeable potent and selective dual ATP and substrate mimetic JNK



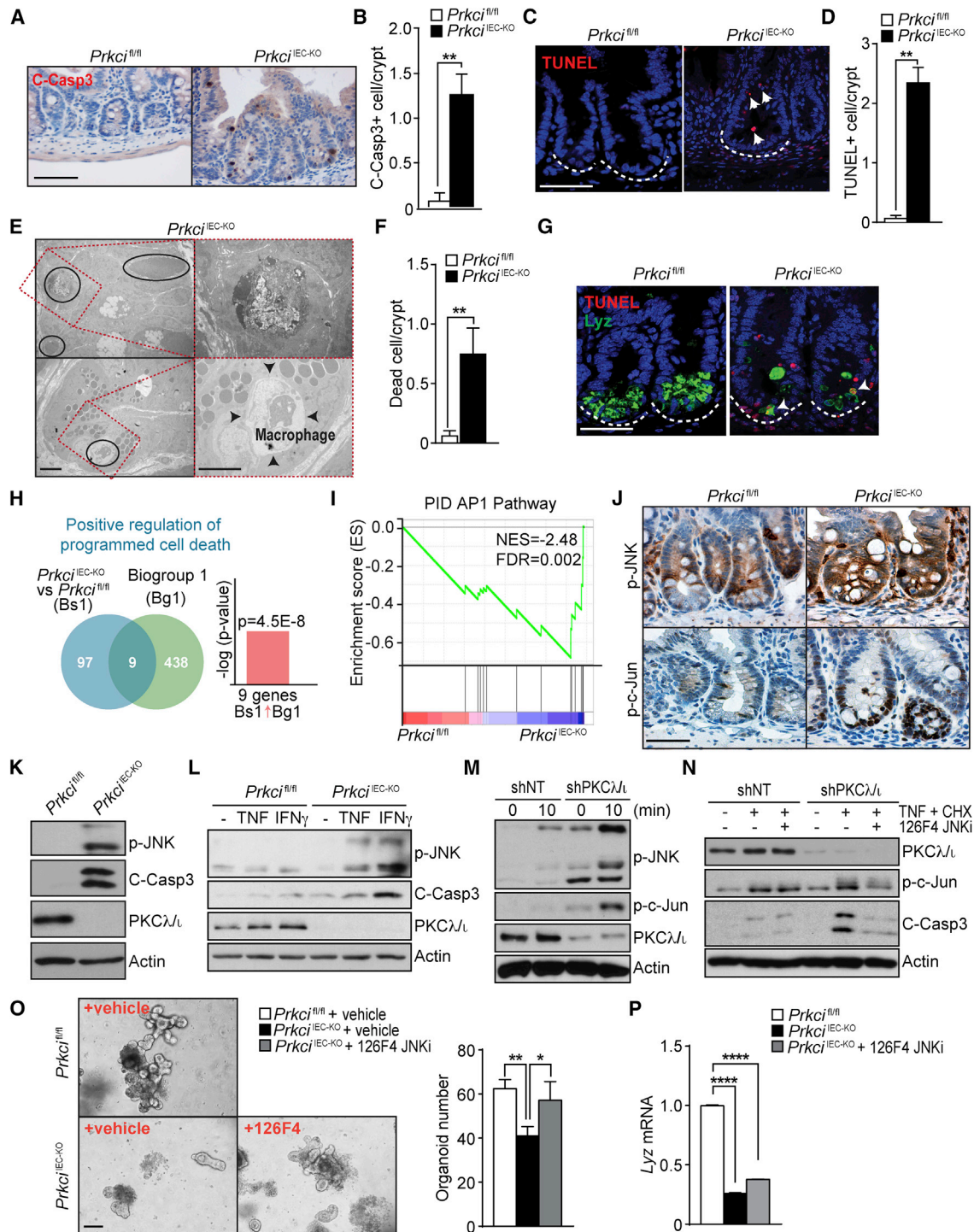


Figure 2. PKCλ/ι Deficiency Increases Intestinal Epithelial Cell Death through JNK Activation

(A) IHC for cleaved caspase-3 (C-Casp3) in *Prkci^{fl/fl}* and *Prkci^{IEC-KO}* small intestinal crypts. (n = 3). Scale bar, 50 μm.
 (B) Quantification of the number of C-Casp3-positive cells per crypt.
 (C) IF for TUNEL (red) of small intestinal sections (n = 3). White dashed line marks the crypt base. Scale bar, 50 μm.
 (D) Quantification of the number of TUNEL-positive cells per crypt.
 (E) EM images showing a number of dead cells (black circles and top, right) in the ileum crypts of *Prkci^{IEC-KO}* mice (n = 3). Infiltrating macrophage engulfed the dead cells (right, bottom). Scale bars, 10 μm.
 (F) Quantification of the number of dead cells per crypt.

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inhibitor termed 126F4. Pre-incubation of PKC λ /I-deficient cells with 126F4 rescued the increased apoptosis (Figures 2N and S2E). 126F4, like the previously reported DJNK1-1 (Barr et al., 2002; Bonny et al., 2001), attenuates JNK activation by interacting with the JIP binding site that is essential for both substrate recognition and activation of JNK by upstream kinases (Barr et al., 2002; Bonny et al., 2001; Stebbins et al., 2008). Furthermore, ex vivo cultures of *Prkci*^{IEC-KO} organoids formed in lower numbers, were smaller and less complex than *Prkci*^{fl/fl} organoids, and displayed impaired viability (Figures 2O and S2F–S2H), which was rescued by incubation with 126F4 (Figure 2O), but no effect on Lyz expression was observed (Figure 2P). Collectively, these results demonstrate that the loss of PKC λ /I in the intestinal epithelium results in impaired Paneth cell homeostasis associated with increased JNK activity and apoptosis of IECs.

Loss of PKC λ /I in the Intestinal Epithelium Results in Increased Inflammation

Paneth cells provide a first line of defense in the epithelial barrier by secreting antimicrobial peptides, which are important for protection against intestinal inflammation (Adolph et al., 2013). This, together with enhanced IEC apoptosis due to increased JNK activity, results in intestinal inflammation. Indeed, NextBio analysis of genes downregulated in *Prkci*^{IEC-KO} crypts revealed significant correlations with the GO terms “defense response” and “MHC class II antigen presentation” (Figure 3A). We also found spontaneous intestinal inflammation with bowel mucosa thickening and increased immune cell infiltration in the small intestines of *Prkci*^{IEC-KO} mice, accompanied by increased expression of inflammatory cytokines (Figures 3B–3D). Histological analysis of colonic sections of *Prkci*^{IEC-KO} mice showed overall mild inflammation with focal areas of immune cell infiltration and microabscess and increased cytokine expression (Figures S3A–S3C). To investigate whether PKC λ /I deficiency sensitizes mice to experimental inflammation in the large intestine, we subjected *Prkci*^{IEC-KO} and control mice to dextran sodium sulfate (DSS). *Prkci*^{IEC-KO} mice lost significantly more weight than *Prkci*^{fl/fl} controls, concomitantly displaying colonic shortening with epithelial destruction and extensive intestinal ulceration (Figures 3E–3G). Reduced Ki67 staining was found in *Prkci*^{IEC-KO} colonic sections, suggesting a defect in the regenerative response (Figure 3G). These results indicate that the loss of PKC λ /I in the intestinal epithelium renders mice highly susceptible to spon-

aneous ileitis as well as spontaneous and experimentally induced colitis.

Because deregulation of intestinal homeostasis and susceptibility to inflammation are often associated with alterations in the commensal bacterial population, we investigated the extent to which bacterial communities were altered in *Prkci*^{IEC-KO} mice. Analysis of *Prkci*^{IEC-KO} mice revealed alterations in the microbiota composition, with increased abundance of Proteobacteria and reduction in Firmicutes (Figures 3H and S3D). Importantly, this observed dysbiosis in *Prkci*^{IEC-KO} mice is consistent with that reported in human IBD patients (Frank et al., 2007). Differences in microbiota distributions were more pronounced after DSS treatment, with *Prkci*^{IEC-KO} mice also showing alterations in Deferribacteres and Bacteroidetes (Figures 3H and S3D). These data suggest that alterations in Paneth cell and increased IEC apoptosis upon PKC λ /I deficiency promote intestinal inflammation through the loss of the mucosal barrier and by modification of the microbiota composition accompanied by bacterial translocation.

PKC λ /I Deficiency Promotes Tumorigenesis via Increased Inflammation and Dysbiosis

Because inflammation is a well-established promoter of intestinal tumorigenesis, we tested whether the loss of PKC λ /I in the intestinal epithelium could contribute to intestinal cancer. Of note, previously published data suggested that PKC λ /I loss in the intestinal epithelium resulted in impaired tumorigenesis in *Apc*^{Min/+} mice (Murray et al., 2009). This is counterintuitive with the tumor promoter role of inflammation and the pro-inflammatory phenotype of *Prkci*^{IEC-KO} mice. To address this question, we crossed *Prkci*^{IEC-KO} mice with *Apc*^{fl/fl} mice to generate *Apc*^{fl/+};*Prkci*^{IEC-KO} mice. Interestingly, *Apc*^{fl/+};*Prkci*^{IEC-KO} mice developed more and larger tumors than *Apc*^{fl/+} control mice in both the small intestine and colon (Figures 4A and 4B). Histological analysis revealed that tumors from *Apc*^{fl/+};*Prkci*^{IEC-KO} mice were often accompanied by ulceration, with a concomitant increase in proliferation and cell death (Figures 4A–4D). Notably, PKC λ /I-deficient tumors also showed increased expression of inflammatory cytokines (Figure 4E), indicating that the increased tumorigenesis could be related to the pro-inflammatory environment created by the lack of PKC λ /I. To further test this hypothesis, we used a colitis-associated CRC model that combines the carcinogen azoxymethane (AOM) and DSS. AOM-DSS-treated *Prkci*^{IEC-KO} mice developed more and larger tumors

(G) Double IF for TUNEL (red) and Lysozyme (Lyz; green) in *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} small intestine. White dashed line marks the crypt base. Scale bar, 50 μ m. (H) NextBio analysis of genes differentially expressed in *Prkci*^{IEC-KO} intestinal crypts and *Prkci*^{fl/fl} controls. Gene signatures corresponding to GO terms “Positive regulation of programmed cell death.”

(I) GSEA enrichment in “PID AP1 Pathway” in crypts from *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice using C2 MSigDB database.

(J) IHC for p-JNK or p-c-Jun of small intestine sections from *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice. (n = 3). Scale bar, 50 μ m.

(K) Immunoblot using antibodies against indicated crypt proteins in *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice.

(L) Immunoblot of small intestinal organoids from *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice using indicated antibodies. Organoids from each genotype were treated with either TNF- α or IFN- γ for 8 hr before collection.

(M) SW480 shNT and shPKC λ /I cells were stimulated with TNF- α and analyzed by immunoblot at indicated time points.

(N) SW480 shNT and shPKC λ /I cells were preincubated with DMSO or JNK inhibitor (126F4), stimulated with TNF- α and cycloheximide (CHX) for 4 hr, and analyzed by immunoblot.

(O and P) Representative pictures and quantification of small intestinal organoids from *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice cultured with or without 126F4 for 3 days (O).

qRT-PCR analysis of mRNA level of *Lyz* in organoids (n = 3) (P). Scale bar, 100 μ m.

Results are shown as mean \pm SEM. *p < 0.05, **p < 0.01. See also Figure S2.

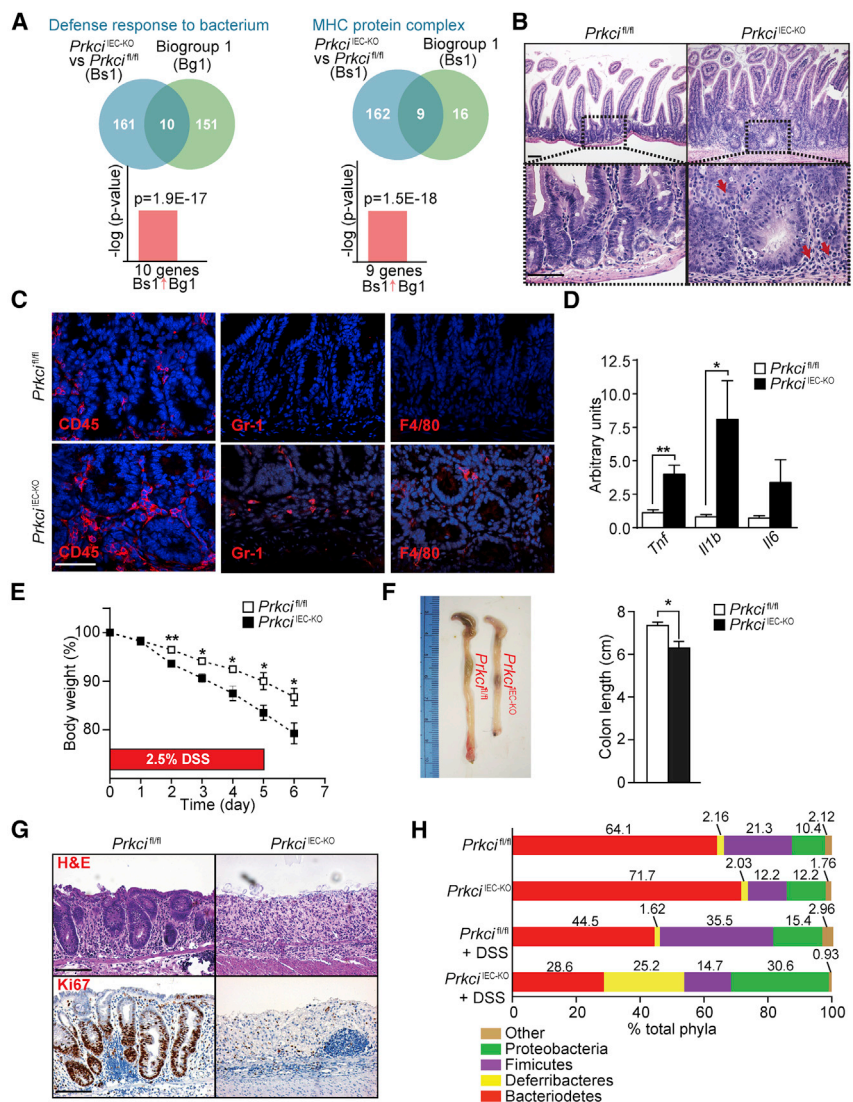


Figure 3. Loss of PKC λ/ι in the Intestinal Epithelium Results in Increased Inflammation

(A) NextBio analysis of genes differentially expressed in *Prkci*^{IEC-KO} intestinal crypts and *Prkci*^{fl/fl} controls. Venn diagrams show the number of common and unique genes in both sets. Gene signatures corresponding to GO terms “defense response” and “MHC protein complex.”

(B) H&E staining of *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} small intestines (n = 6). Red arrows point to immune cell infiltrates.

(C) IF staining for CD45, Gr-1, or F4/80 in *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} small intestinal sections (n = 3).

(D) qRT-PCR analysis of mRNA levels of inflammatory cytokines in ileum crypts of *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice (n = 6).

(E–G) Percentage of change in body weight (E), representative pictures and quantification of colon length (F), and H&E and Ki67 staining (G) of *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} colon sections (n = 4) subjected to a DSS-induced colitis model.

(H) Phylum-level analysis of microbiota by 16S rDNA pyrosequencing of fecal pellets at basal conditions and after DSS treatment in *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice (n = 8). Scale bars, 50 μ m.

Results are shown as mean \pm SEM. *p < 0.05, **p < 0.01. See also Figure S3.

role for PKC λ/ι as a non-cell-autonomous tumor suppressor in inflammation-induced intestinal tumorigenesis. Importantly, the reduction in inflammation and cell death promoted by antibiotic treatment did not restore Paneth cells to normal levels in *Apc*^{fl/+}; *Prkci*^{IEC-KO} mice (Figures 4I, 4J, and S4E). This demonstrated that PKC λ/ι deficiency in the intestinal epithelium results in defects in Paneth cell homeostasis independent of cell death and the presence of inflammation in the crypt microenvironment. This is in agreement with data in Fig-

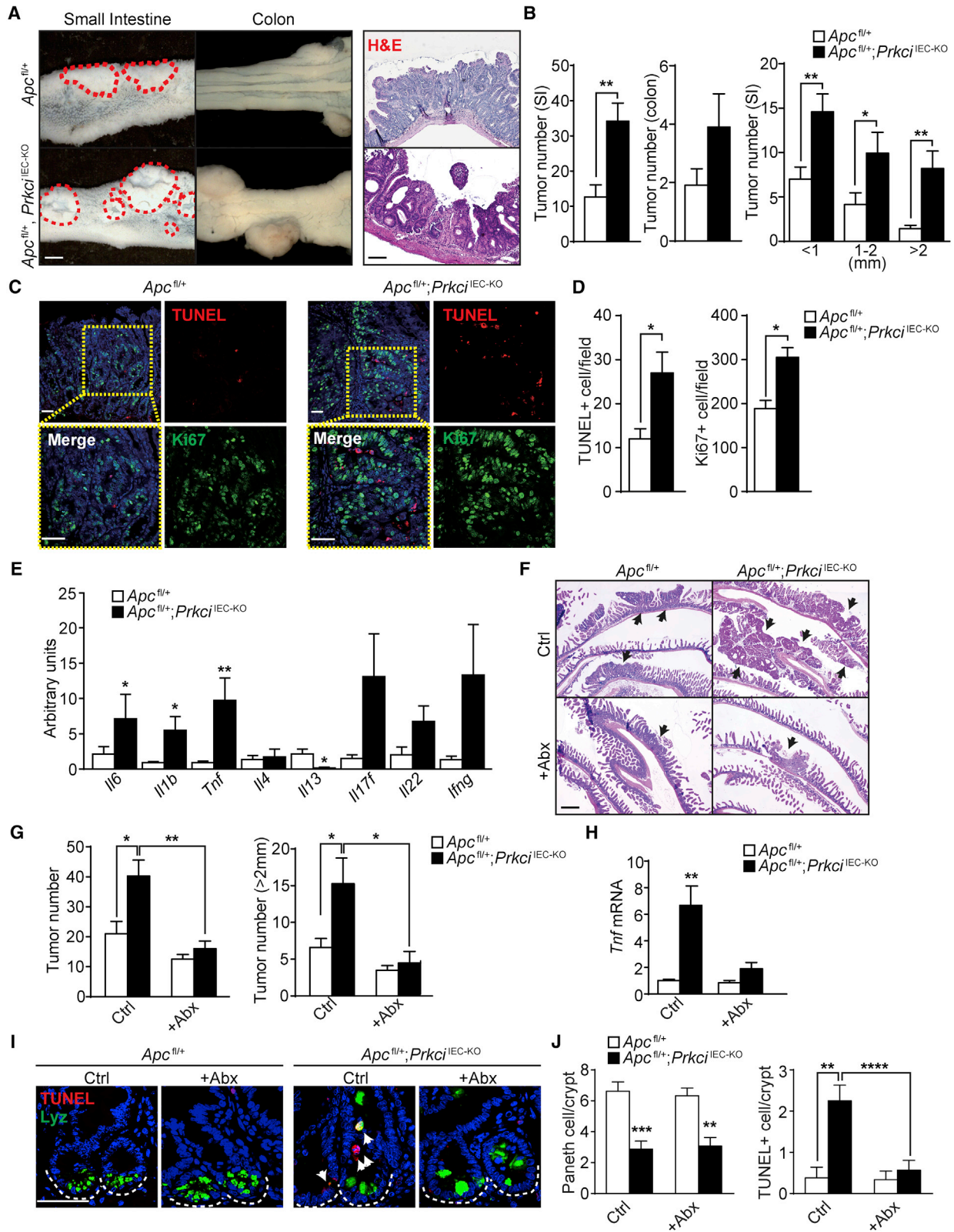
ures 2O and 2P demonstrating that JNK inhibition in organoids rescued cell death, but not Paneth cell differentiation defects.

than identically treated *Prkci*^{fl/fl} mice, which also displayed higher rates of proliferation and cell death (Figures S4A–S4D). Collectively, these results are consistent with a model whereby the loss of PKC λ/ι in the intestinal epithelium impairs Paneth cell function and increases IEC apoptosis, resulting in barrier disruption, commensal infiltration, and severe inflammation, which promotes intestinal tumorigenesis. To rigorously test this hypothesis, we administered a cocktail of broad-spectrum antibiotics to mice of both genotypes and determined the effect on tumor development. Interestingly, antibiotic treatment impaired the increased tumorigenesis phenotype of *Apc*^{fl/+}; *Prkci*^{IEC-KO} mice, which showed a reduction in the number and size of tumors compared to untreated mice (Figures 4F and 4G). This effect was accompanied by a decrease in *Tnf* expression (Figure 4H). Similar results were obtained when the colons of these mice were analyzed (Figures S4F–S4H). These results established that antibiotic treatment diminished the pro-tumorigenic inflammatory conditions of PKC λ/ι -deficient intestines, supporting a

role for PKC λ/ι as a non-cell-autonomous tumor suppressor in inflammation-induced intestinal tumorigenesis. Importantly, the reduction in inflammation and cell death promoted by antibiotic treatment did not restore Paneth cells to normal levels in *Apc*^{fl/+}; *Prkci*^{IEC-KO} mice (Figures 4I, 4J, and S4E). This demonstrated that PKC λ/ι deficiency in the intestinal epithelium results in defects in Paneth cell homeostasis independent of cell death and the presence of inflammation in the crypt microenvironment. This is in agreement with data in Fig-

PKC λ/ι -Deficiency Impairs Paneth Cell Differentiation through Atoh1

Because PKC λ/ι 's effects on Paneth cell differentiation and intestinal homeostasis were not a consequence of increased cell death, we next asked whether Paneth cell defects in *Prkci*^{IEC-KO} mice were actually due to impaired differentiation or to defective maintenance. To address this question, we characterized Paneth cell alterations in *Prkci*^{IEC-KO} mice by double staining for Lyz and Alcian blue (AB), a marker of goblet cells. While in *Prkci*^{fl/fl} intestines Lyz expression is localized in the crypt base and does not merge with AB, in *Prkci*^{IEC-KO} intestines, a significant number of Lyz⁺ cells were found in the villus and upper crypt co-stained for Lyz and AB (Figures 5A and 5B). These data suggest that PKC λ/ι deficiency leads to a failure of intestinal secretory progenitor cells to properly differentiate into Paneth



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cells and to default to an intermediate goblet cell-like phenotype. In keeping with this hypothesis, the expression levels of *Atoh1* and *Gfi1* were lower in the crypts from *Prkci*^{IEC-KO} mice (Figure 5C). *Atoh1* and *Gfi1* are critical transcription factors for the differentiation of Paneth cells (Shroyer et al., 2005; Yang et al., 2001). To test whether *PKCλ/ι* might also regulate Paneth cell maintenance or survival after differentiation, we crossed *Prkci*^{fl/fl} mice with a *Defa6-cre* mouse line (Adolph et al., 2013) to create *Prkci*^{PC-KO}, in which *PKCλ/ι* is selectively deleted in terminally differentiated Paneth cells. We confirmed the exclusive expression of this Cre line in Paneth cells by crossing with the Rosa-LacZ reporter (Figure S5A). Deletion of *PKCλ/ι* in *Prkci*^{PC-KO} Paneth cells was also verified by double staining of *PKCλ/ι* and *Lyz* (Figure S5B). Importantly, no defects were found in Paneth cell numbers or *Lyz* content in *Prkci*^{PC-KO} mice (Figures S5C–S5E). Of note, these mice did not show spontaneous enteritis or increased sensitivity to colitis (Figures S5F–S5H). These results strongly suggest that *PKCλ/ι* is important for proper Paneth cell differentiation, but not for Paneth cell maintenance or survival, in terminally differentiated cells.

A critical event in Paneth cell differentiation is Notch inactivation in precursor cells (Fre et al., 2005; van Es et al., 2005; Yin et al., 2014) (Figure 5D). We next determined whether inactivation of Notch signaling could rescue the defect in Paneth cell differentiation caused by *PKCλ/ι* deficiency. For this, we incubated organoids of both genotypes with DAPT, a selective and widely used γ -secretase inhibitor of the Notch pathway (Dovey et al., 2001; Yin et al., 2014). Interestingly, incubation with DAPT led to a robust induction of *Lyz* in *Prkci*^{fl/fl}, but not *Prkci*^{IEC-KO}, organoids (Figure 5E). The same results were obtained when the levels of *Defa6*, another marker of Paneth cell differentiation, or those of goblet (*Muc2*) or enteroendocrine (*Chga*) cells were determined (Figures 5E and 5F). In contrast, *Alpi*, which is a marker of enterocytes, was not affected by DAPT treatment of *PKCλ/ι*-deficient organoids (Figure 5F). Notably, the addition of DAPT did not activate the expression of either *Atoh1* or *Gfi1* in *PKCλ/ι*-deficient organoids, although it induced a robust response in *Prkci*^{fl/fl} organoids (Figure 5G). Collectively, these results demonstrate that *PKCλ/ι* is required for Paneth cell differentiation, most probably through the control of the transcriptional regulation of *Atoh1* and *Gfi1* upon Notch inactivation.

Epigenetic Control of *Atoh1* Expression by *PKCλ/ι*

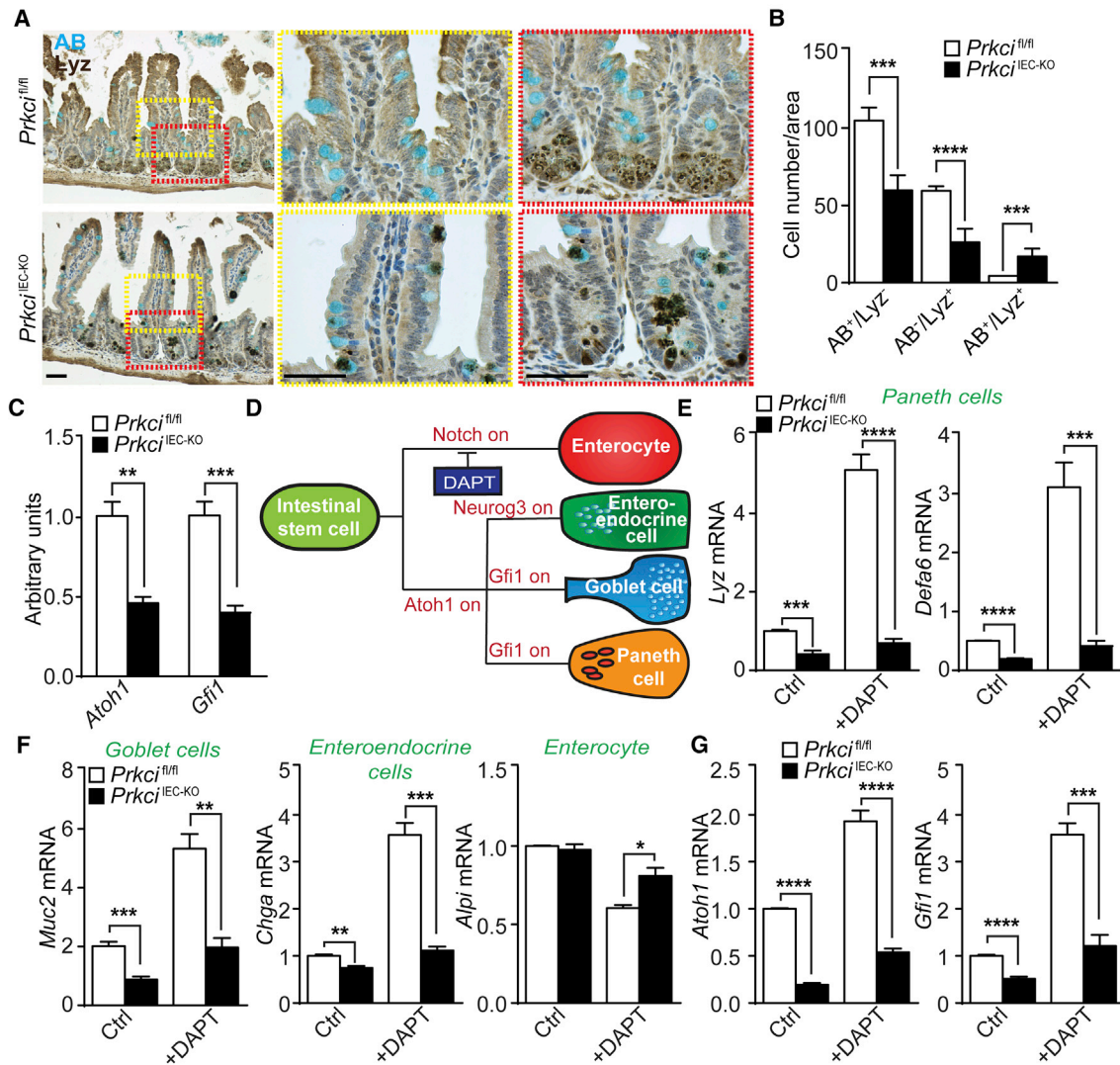
GSEA of the transcriptomic experiment described above provided an important clue in understanding the precise mecha-

nisms whereby *PKCλ/ι* regulates *Atoh1* expression. Interestingly, a category that was also significantly upregulated in *PKCλ/ι*-deficient crypts was “SUZ12_TARGETS” (Figure 6A). SUZ12 is a critical component of the polycomb repressive complex 2 (PRC2), which is well known for its ability to repress cell differentiation and regulate self-renewal in embryonic stem cells via histone methylation to produce H3K27me3 (Margueron and Reinberg, 2011). We hypothesized that epigenetic modifications controlled by *PKCλ/ι* could regulate *Atoh1* expression. Importantly, ENCODE interrogation of the regulatory region of both *Atoh1* and *Gfi1* genes revealed the potential for recruitment of SUZ12 and EZH2, the enzymatic subunit of the PRC2 complex, along with the repression marker H3K27me3 (Figure 6B). Conceivably, *PKCλ/ι* could influence PRC2 activity to control *Atoh1* expression. To test this possibility, we performed chromatin immunoprecipitation (ChIP) analysis in crypts from *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice and found that both *Atoh1* and *Gfi1* regulatory regions were highly occupied by EZH2, which correlated with enrichment of the repression marker H3K27me3 (Figure 6C). Immunoblot analysis demonstrated increased levels of EZH2 in *PKCλ/ι*-deficient crypts (Figure 6D). Similar results were obtained when the levels of EZH2 were determined in 293 cells in which *PKCλ/ι* had been knocked out by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing (Figure 6E). Notably, double IF analysis of intestinal samples from both mouse genotypes showed a strong accumulation of H3K27me3 and EZH2 signals in the crypts of *Prkci*^{IEC-KO} mice (Figure 6F).

In vitro kinase assay using purified hemagglutinin (HA)-tagged EZH2 and recombinant *PKCλ/ι* demonstrated that *PKCλ/ι* robustly phosphorylated EZH2 (Figure 6G). To map the *PKCλ/ι* phosphorylation sites in EZH2, we used titanium dioxide (TiO₂)-based phosphopeptide enrichment on enzymatic digests of in vitro phosphorylation reactions followed by high-performance liquid chromatography tandem mass spectrometry (MS/MS) analysis. Using this approach, we identified T487 as a major *PKCλ/ι* phosphorylation site and several sites of low abundance such as S690 and T345 (Figure S6). T487 and T345 are reported as sites important for protein degradation (Wu and Zhang, 2011). This suggests that *PKCλ/ι* might control EZH2 protein stability. Consistently, EZH2 was significantly more stable in cycloheximide-treated *PKCλ/ι*-deficient cells (Figure 6H). These data strongly suggest that loss of EZH2 phosphorylation upon *PKCλ/ι* deficiency increases EZH2 stability and H3K27me3 levels, which represses *Atoh1* and *Gfi1* expression and Paneth cell

Figure 4. Increased Inflammation and Dysbiosis Induced by *PKCλ/ι* Deficiency Promotes Tumorigenesis

- (A) Macroscopic images and H&E staining of tumors (red dashed circles) in small intestine or colon from *Apc*^{fl/+} and *Apc*^{fl/+};*Prkci*^{IEC-KO} mice (n = 10).
 (B) Quantification of the total tumor numbers and stratification of tumor numbers according to size (n = 10).
 (C) Double IF for TUNEL (red) and Ki67 (green) in small intestine tumors of *Apc*^{fl/+} and *Apc*^{fl/+};*Prkci*^{IEC-KO} mice (n = 3).
 (D) Quantification of the number of TUNEL- or Ki67-positive cells per field.
 (E) qRT-PCR analysis of inflammatory cytokine mRNA levels in small intestinal tumors from *Apc*^{fl/+} and *Apc*^{fl/+};*Prkci*^{IEC-KO} mice (n = 6).
 (F) H&E staining of small intestine from *Apc*^{fl/+} and *Apc*^{fl/+};*Prkci*^{IEC-KO} mice with or without antibiotic treatment (n = 5). Black arrows point to tumors.
 (G) Quantification of the number of all tumors (left) or large tumors (right; size > 2 mm) in *Apc*^{fl/+} and *Apc*^{fl/+};*Prkci*^{IEC-KO} mice with or without antibiotic (Abx) treatment (n = 5–7).
 (H) qRT-PCR analysis of *Tnf* mRNA levels in the tumors of *Apc*^{fl/+} and *Apc*^{fl/+};*Prkci*^{IEC-KO} small intestine (n = 6).
 (I) Double IF for lysozyme (*Lyz*; green) and TUNEL (red) in non-tumor lesions of *Apc*^{fl/+} and *Apc*^{fl/+};*Prkci*^{IEC-KO} small intestine (n = 3).
 (J) Quantification of the number of *Lyz*-positive Paneth cells and TUNEL-positive cells per crypt. Scale bars, 50 μ m.
 Results are shown as mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001. See also Figure S4.

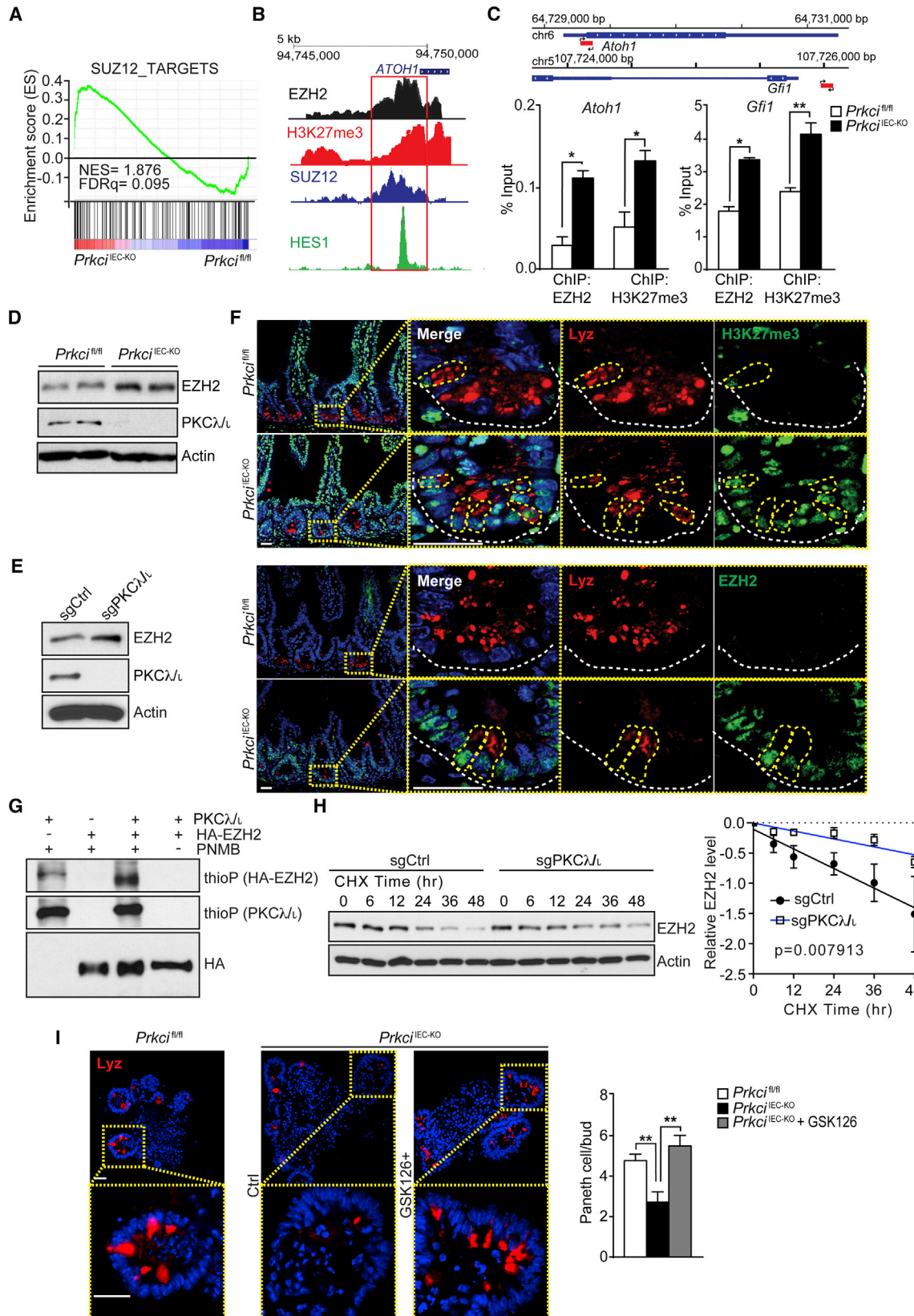


differentiation. In support of this model, treatment with GSK126, an inhibitor of EZH2 activity, resulted in upregulation of the Paneth cell population in *Prkci^{IEC-KO}* organoids (Figure 6I). Collectively, these results demonstrate that PKC λ 1 is required for Paneth cell differentiation through the regulation of PRC2 activity and that this event is necessary to allow *Atoh1* and *Gfi1* transcription.

PKC λ 1 Is Expressed in Human Intestinal Paneth Cells, and Its Expression Decreases with Progression of Crohn's Disease

Paneth cell dysfunction and ileal inflammation have been linked to the etiology of IBD (Cadwell et al., 2008). Therefore, to inves-

tigate the relevance of PKC λ 1 to Paneth cell function in human patients, we used immunohistochemistry (IHC) for PKC λ 1 to analyze 40 ileum tissue sections derived from patients with CD. Expression of PKC λ 1 was first determined in normal mucosa from uninvolved proximal margins containing little or no inflammation. Importantly, PKC λ 1 staining strongly localized with that of Lyz, as demonstrated by double IF (Figure S7A). This indicated that PKC λ 1 expression, similar to our findings in mice, was enriched in human Paneth cells. To study the expression of PKC λ 1 upon disease progression, samples were subdivided by a pathologist, blinded to sample origins, into different categories (mild, moderate, and severe) according to the



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severity of inflammation in the intestinal mucosa. Notably, there was a gradual reduction in PKC $\lambda/1$ with CD progression, with minimal expression in the most severe group (Figures 7A and 7B). Furthermore, active inflammatory lesions in the intestinal samples of CD patients showed that low PKC $\lambda/1$ /Lyz⁺ cells were not restricted to the crypt base and displayed a disorganized and diffuse pattern of Lyz expression not confined to the granule, as opposed to that in normal mucosa (Figures 7C, top, and S7A). In addition, a large number of TUNEL-positive cells were observed in the crypts of active lesions correlating with low PKC $\lambda/1$ expression (Figure 7C, bottom). Interestingly, double Lyz and AB staining of moderate-to-severe CD patients demonstrated a large number of mislocalized immature Paneth cells that were positive for both Lyz and AB (Figure 7D). These findings suggest a defect in Paneth cells in CD samples similar to that observed in *Prkci*^{IEC-KO} mice. Likewise, CD crypts with low PKC $\lambda/1$ levels showed enhanced expression of H3K27me3 similar to the phenotype of PKC $\lambda/1$ -deficient mice (Figure 7E).

To expand on these observations, we analyzed *PRKCI* mRNA expression in public gene datasets of CD and UC. Consistent with the results of the IHC of CD samples, PKC $\lambda/1$ was significantly downregulated in CD and UC samples, as compared to healthy controls, in all seven datasets analyzed (Figures 7F and S7B). Furthermore, there was a significant positive correlation between *PRKCI* and *ATOH1* mRNA levels in three different datasets of IBD samples (Figure 7G), in agreement with a critical role for PKC $\lambda/1$ in Paneth cell differentiation through the control of Atoh1 expression. We next performed GSEA of correlation profiles in the IBD clinical dataset (GSE59071) that included the larger number of samples from both CD and UC patients in which *PRKCI* mRNA was downregulated. Analyses with the H compilation of MSigDB (Broad Institute) revealed that *PRKCI* levels inversely correlated with gene signatures related to inflammation (Figures 7H and 7I), consistent with a role for PKC $\lambda/1$ in controlling intestinal homeostasis.

Because PKC $\lambda/1$ -deficient mice had an inflammatory and pro-tumorigenic phenotype, we next investigated the role of PKC $\lambda/1$ in human CRC in the context of inflammation. For this, we carried out a molecular concept map analysis of CRC microarray data in the Oncomine database (Rhodes et al., 2007) using signatures of *PRKCI*-correlated or *PRKCI*-anticorrelated genes derived from the IBD clinical cohort (GSE59071). A heatmap visualization of significant comparisons showed a strong overlap of *PRKCI*-correlated genes with those underexpressed in colon and colo-

rectal tumors versus normal samples in advanced clinical stage versus earlier stages and in patients with poor versus good prognoses (Figure S7C). Accordingly, *PRKCI*-anticorrelated genes showed significant overlap with those found to be overexpressed in these same categories (Figure S7C). Furthermore, Kaplan-Meier survival analysis of CRC patients stratified according to *PRKCI* expression revealed that low *PRKCI* levels correlated with significantly worse patient survival (Figure 7J). All of these results support an unanticipated role of *PRKCI* as a non-cell-autonomous tumor suppressor in the context of colon cancer associated with inflammation.

DISCUSSION

Alterations in intestinal epithelial homeostasis contribute to dysbiosis and intestinal inflammation and are clearly involved in intestinal cancer initiation and progression (Medema and Vermeulen, 2011; Peterson and Artis, 2014). Paneth cells are critical for creating an anti-microbial and nurturing environment that aids in the survival and function of crypt cells and maintenance of the intestinal barrier (Clevers and Bevins, 2013; Sato et al., 2011). Here, we show that PKC $\lambda/1$ is expressed in the whole intestinal epithelium and prominently in Paneth cells and demonstrate that it plays a dual role by promoting Paneth cell differentiation and the survival of crypt cells. Thus, PKC $\lambda/1$ deficiency, by impairing Paneth cell differentiation, triggers an inflammatory response that is further amplified by increased apoptosis in the whole intestinal epithelium both in the small intestine and colon, and that is driven by the microbiota. Importantly, we also showed that human CD patients displayed reduced PKC $\lambda/1$ levels that correlated with impaired Paneth cell development, IEC death, and intestinal inflammation.

Germane to these observations is the fact that PKC $\lambda/1$ is required, in a cell-autonomous manner, for the optimal transcriptional activation of the two master regulators of Paneth cell differentiation, Atoh1 and Gfi1, through their epigenetic control upon Notch inactivation. This explains why the phenotype of *Prkci*^{IEC-KO} shared some characteristics with Atoh1 and Gfi1 knockout (KO) mice (Shroyer et al., 2005, 2007; Yang et al., 2001). In this regard, Atoh1 KO mice display a complete loss of all secretory lineages, whereas Gfi1 KO mice only have a partial reduction in the number of Paneth and goblet cells, with a concomitant increase in enteroendocrine cells (Shroyer et al., 2005, 2007; Yang et al., 2001). In contrast, *Prkci*^{IEC-KO} mice,

Figure 6. PKC $\lambda/1$ Regulates Atoh1 by Controlling EZH2 Phosphorylation and Stability

(A) GSEA of SUZ12 targets in crypts from *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice using C2 MSigDB database.

(B) ENCODE schematic of epigenetic marks in *ATOH1* gene.

(C) Primer design for CHIP-qPCR analysis of *Atoh1* and *Gfi1* occupancy of EZH2 and H3K27me3. CHIP assay was performed with anti-EZH2 or anti-H3K27me3 antibodies. Co-immunoprecipitated DNA was examined using qPCR with primers specific for the *Atoh1* or *Gfi1* regulatory region (n = 3).

(D and E) Immunoblot analysis of EZH2 in crypt proteins from *Prkci*^{fl/fl} and *Prkci*^{IEC-KO} mice (D) or 293 PKC $\lambda/1$ -KO cells generated by the CRISPR/CAS9 system (E).

(F) Double IF for lysozyme (Lyz; red) and H3K27me3 (top) or EZH2 (bottom) (green). White dashed line marks the crypt base (n = 3).

(G) In vitro phosphorylation of HA-tagged EZH2 immunoprecipitates by baculovirus-expressed recombinant PKC $\lambda/1$ with ATP- γ S followed by PNBM alkylation and immunoblotting for the indicated proteins.

(H) CRISPR/CAS9-mediated 293 PKC $\lambda/1$ -KO or control cells were incubated with cycloheximide (CHX) and protein stability was determined by immunoblotting at indicated time points. EZH2 protein levels were normalized to actin (n = 5).

(I) Small intestinal organoids from WT and *Prkci*^{IEC-KO} mice were cultured and treated with or without GSK126 for 5 days and analyzed by IF for Lyz (n = 4). Representative pictures and quantification are shown. Scale bars, 25 μ m.

Results are shown as mean \pm SEM. **p < 0.01. See also Figure S6.

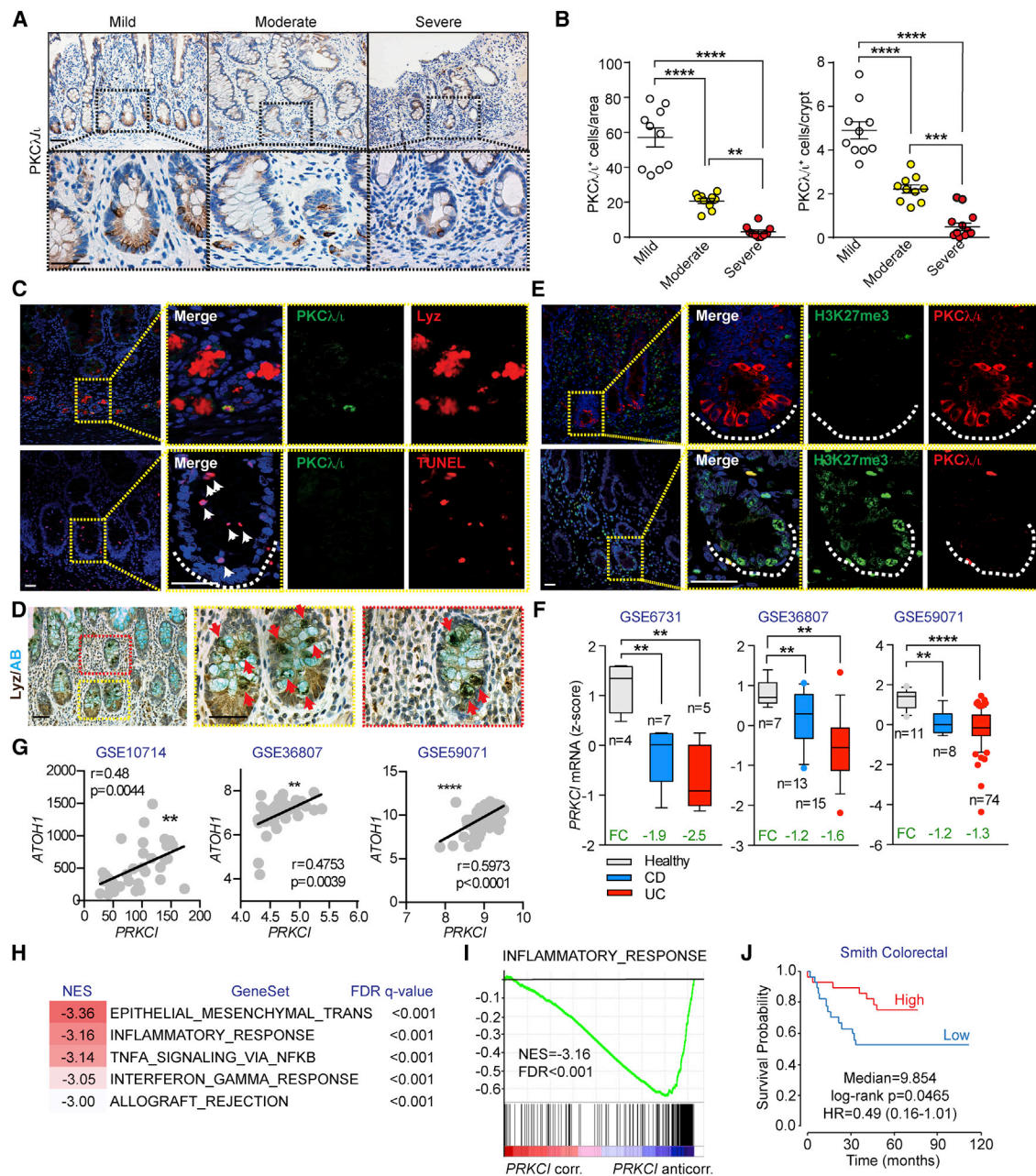


Figure 7. PKCλ/ι Levels Are Downregulated in Clinical Samples from Inflammatory Bowel Disease Patients

For a Figure360 author presentation of Figure 7, see <http://dx.doi.org/10.1016/j.celrep.2016.08.054#mmc3>

(A) IHC for PKCλ/ι in ileum sections from patients with CD. Subgroups were assigned according to severity of the disease (n = 10 for each subgroup). Scale bars, 50 μm.

(B) Quantification of PKCλ/ι-positive cells per area or per crypt of tissue sections in (A).

(C) Double IF for lysozyme (top; Lyz, red) or TUNEL (bottom; red) and PKCλ/ι (green). White arrowheads point to TUNEL-positive cells. White dashed line marks the crypt base (n = 3). Scale bars, 25 μm.

(D) Double staining for Alcian blue (AB) and Lyz in ileum sections from patients with CD (n = 3). Red arrowheads point the colocalization of AB and Lyz. Scale bars, 50 μm.

(E) Double IF for PKCλ/ι (red) and H3K27me3 (green) in ileum sections of patients with CD (n = 3). White dashed line marks the crypt base. Scale bars, 25 μm.

(F) PRKCI mRNA levels in IBD patient samples. Data were collected from public datasets of gene expression.

(G) Pearson correlation analysis of gene expression between ATOH1 and PRKCI in three different IBD datasets.

(H) GSEA of the correlation profile for PRKCI in an IBD clinical cohort (GSE59071) using the “Hallmarks” compilation from Molecular Signature Database (MSigDB, Broad Institute).

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although dramatically affecting the levels of mature Paneth cells, did not show their complete ablation, consistent with the fact that PKC $\lambda/1$ inactivation resulted in the reduction, but not complete loss, of Atoh1 and Gfi1 expression. Furthermore, proof that Paneth cell defects are not secondary to the increased sensitivity of IECs to apoptosis or inflammation is that inhibition of JNK activity in organoid cultures rescued the increased apoptosis associated with PKC $\lambda/1$ ablation but did not rescue the defect in Paneth cell differentiation. Likewise, antibiotic treatment did not restore normal Paneth cell homeostasis in vivo, although it completely abolished intestinal inflammation, which is in keeping with the notion that PKC $\lambda/1$ is a cell-autonomous epigenetic regulator of Paneth cell differentiation. Interestingly, treatment of PKC $\lambda/1$ -deficient organoids with a pharmacological PRC2 inhibitor fully normalized Paneth cell levels. Therefore, we propose here that epigenetic modification drugs, such as EZH2 inhibitors, could be a valid therapeutic strategy for IBD and intestinal cancer.

In this regard, our results also have important implications beyond the biology of intestinal inflammation and resolve an important conundrum in the PKC field by definitively establishing a non-cell-autonomous tumor suppressor role for PKC $\lambda/1$ in intestinal carcinogenesis. Intriguingly, previous data suggested that loss of PKC $\lambda/1$ in the intestinal epithelium prevented tumorigenesis in the *Apc*^{Min/+} mouse model (Murray et al., 2009), which correlated with more inflammation in the DSS model (Calcagno et al., 2011). These were paradoxical results if it is taken into consideration that inflammation is a tumor-promoter state and a risk factor for intestinal cancer in human patients (Karin and Clevers, 2016). Our results reported here resolve this puzzle by showing that the loss of PKC $\lambda/1$ in the epithelium results in more, not less, tumorigenesis. Of note, this effect is detected both under basal and DSS-induced conditions and, importantly, is abrogated in mice in which the microbiota has been obliterated by antibiotic treatment. We suggest that caution should be taken when considering the pharmacological inhibition of PKC $\lambda/1$ as an anti-cancer strategy, since it will result in an enhanced risk of CRC.

EXPERIMENTAL PROCEDURES

A detailed description of the experimental procedures utilized in this work can be found in [Supplemental Experimental Procedures](#). Primers used are described in [Table S1](#).

Mice

All mouse strains were generated in a C57BL/6 background. All mice were born and maintained under pathogen-free conditions. Animal handling and experimental procedures conformed to institutional guidelines (SBP Medical Discovery Institute Institutional Animal Care and Use Committee).

CD Patient Samples

Surgically resected specimens were obtained from CD patients who had been admitted to Hyogo College of Medicine in Japan. Written informed consent

was obtained from patients with the protocol approved by the ethics committee of Hyogo College of Medicine. De-identified samples were sent to SBP Medical Discovery Institute and used for histological analyses. The study was approved by the ethics committee of SBP Medical Discovery Institute.

Statistical Analyses

All the statistical tests are justified for every figure. Data are presented as the mean \pm SEM. Significant differences between groups were determined using a Student's *t* test (two-tailed unpaired) when the data were normally distributed (D'Agostino test). If the data were not normally distributed, a Mann-Whitney test was used. The significance level for statistical testing was set at $p < 0.05$. All experiments were performed at least two or three times.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE84118.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.08.054>.

AUTHOR CONTRIBUTIONS

M.T.D.-M. and J.M. devised and coordinated the project. Y.N., M.R.-C., N.N., V.L., L.E., A.C., and S.K.D. performed the experiments. M.L., H.I., M.P., and R.S.B. provided materials that made the study possible. Y.N., M.R.-C., V.L., L.E., A.C., S.P., M.T.D.-M., and J.M. analyzed the data. Y.N., M.T.D.-M., and J.M. designed the experiments and wrote the manuscript with help from S.P., M.P., and R.S.B.

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(I) Enrichment plot corresponding to "INFLAMMATORY_RESPONSE" gene set of GSEA in (H).

(J) Kaplan-Meier plot generated by median split of colorectal cancer patients from "Smith Colorectal" dataset according to their *PRKCI* expression (Precog, Stanford).

Results are shown as mean \pm SEM. ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. See also [Figure S7](#).

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