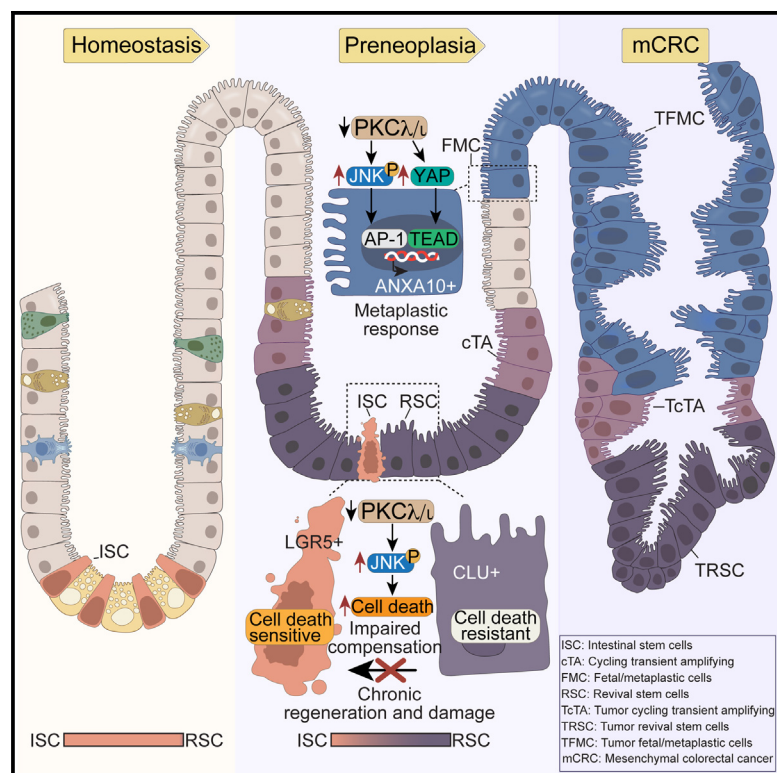


Developmental Cell

Epithelial aPKC deficiency leads to stem cell loss preceding metaplasia in colorectal cancer initiation

Graphical abstract



Authors

Hiroto Kinoshita,
Anxo Martinez-Ordoñez,
Tania Cid-Diaz, ..., Giorgio Inghirami,
Maria T. Diaz-Meco, Jorge Moscat

Correspondence

mtd4001@med.cornell.edu (M.T.D.-M.),
jom4010@med.cornell.edu (J.M.)

In brief

Kinoshita and Martinez-Ordoñez et al. demonstrate that aPKC loss is an early event in the initiation of colorectal cancer through the conventional and serrated pathways. This is associated with impaired intestinal stem cells, preceding the activation of the revival and metaplastic cell programs through a JNK-driven AP-1/YAP cascade.

Highlights

- aPKC deficiency acutely drives intrinsic stem cell loss in the intestinal epithelium
- Stem cells are lost before metaplasia and in aPKC-low dysplasia and CRC
- Revival stem and fetal metaplasia cells are two spatiotemporally distinct populations
- JNK activation by low aPKC leads to stem cell loss and metaplasia through AP-1/YAP



Article

Epithelial aPKC deficiency leads to stem cell loss preceding metaplasia in colorectal cancer initiation

Hiroto Kinoshita,^{1,7} Anxo Martinez-Ordoñez,^{1,7} Tania Cid-Díaz,¹ Qixiu Han,¹ Angeles Duran,¹ Yu Muta,^{1,2} Xiao Zhang,¹ Juan F. Linares,¹ Yuki Nakanishi,² Hiroaki Kasashima,³ Masakazu Yashiro,³ Kiyoshi Maeda,³ Ana Albaladejo-Gonzalez,^{4,5} Daniel Torres-Moreno,^{4,6} José García-Solano,^{4,5} Pablo Conesa-Zamora,^{4,6} Giorgio Inghirami,¹ Maria T. Diaz-Meco,^{1,*} and Jorge Moscat^{1,8,*}

¹Department of Pathology and Laboratory Medicine and Sandra and Edward Meyer Cancer Center, Weill Cornell Medicine, New York, NY 10065, USA

²Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan

³Department of Gastroenterological Surgery, Osaka Metropolitan University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka city 545-8585, Japan

⁴Department of Histology and Pathology, Faculty of Life Sciences, Universidad Católica de Murcia (UCAM), 30107 Murcia, Spain

⁵Department of Pathology, Santa Lucía General University Hospital (HGUSL), Calle Mezquita sn, 30202 Cartagena, Spain

⁶Department of Clinical Analysis, Santa Lucía General University Hospital (HGUSL), Calle Mezquita sn, 30202 Cartagena, Spain

⁷These authors contributed equally

⁸Lead contact

*Correspondence: mtd4001@med.cornell.edu (M.T.D.-M.), jom4010@med.cornell.edu (J.M.)

<https://doi.org/10.1016/j.devcel.2024.05.001>

SUMMARY

The early mechanisms of spontaneous tumor initiation that precede malignancy are largely unknown. We show that reduced aPKC levels correlate with stem cell loss and the induction of revival and metaplastic programs in serrated- and conventional-initiated premalignant lesions, which is perpetuated in colorectal cancers (CRCs). Acute inactivation of PKC λ/ι *in vivo* and in mouse organoids is sufficient to stimulate JNK in non-transformed intestinal epithelial cells (IECs), which promotes cell death and the rapid loss of the intestinal stem cells (ISCs), including those that are LGR5⁺. This is followed by the accumulation of revival stem cells (RSCs) at the bottom of the crypt and fetal-metaplastic cells (FMCs) at the top, creating two spatiotemporally distinct cell populations that depend on JNK-induced AP-1 and YAP. These cell lineage changes are maintained during cancer initiation and progression and determine the aggressive phenotype of human CRC, irrespective of their serrated or conventional origin.

INTRODUCTION

Colorectal cancer (CRC), the second leading cause of cancer-related deaths in the USA,¹ typically develops from two different histological premalignant states: conventional adenomas (CAs) and serrated adenomas (SAs).^{2,3} SA-derived CRCs can be deficient or proficient in mismatch repair (MMR) proteins, either microsatellite instable (MSI) or stable (MSS), and might be accompanied by *BRAF* or, to a lesser extent, *KRAS* mutations.^{4–6} By contrast, CAs are always driven by Adenomatous polyposis coli (APC) inactivation with the consequent constitutive stimulation of the WNT pathway and are associated with mutations in several drivers, including p53 and, in many cases, *KRAS*.⁷ These tumors are MMR-proficient, MSS, and often present with chromosomal instability (CIN).^{5,7} Several mouse models recapitulate at least partially different aspects of both mechanisms, providing critical insights into how tumors progress once initiated by the loss of suppressors like APC and p53 or the expression of onco-

genes such as mutant *KRAS*, *BRAF*, or *NOTCH*.^{8–10} However, information on the chronological sequence of spontaneous mechanisms of initiation before the alterations in the expression of tumor suppressors or oncogenes occur must be better understood. This is important for identifying potential targets for prevention before the initial neoplastic lesions acquire a malignant phenotype. In that regard, a hypothesis to explain how tumors initiate through the serrated pathway posits that this is driven by a poorly characterized mechanism of metaplastic alteration of the cells at the top of the crypt that creates a preneoplastic serrated event in response to a still undefined chronic tissue-damaging signal.^{11,12} By contrast, neoplastic initiation through the CA pathway is characterized by a process involving the activation and expansion of the stem cell compartment at the bottom of the crypt triggered by APC inactivation.^{11,12} Given the tremendous heterogeneity of CRC, these two alternative initiation mechanisms are likely an oversimplification of more complex cellular and molecular processes.

We have recently reported that the simultaneous genetic inactivation of the two atypical protein kinase C (aPKC) isoforms, PKC λ/ι and PKC ζ , in the mouse intestinal epithelium results in the spontaneous development of SAs that progress to advanced cancer without additional exogenously induced genotoxic or oncogenic insults.¹³ The interrogation of several gene expression datasets and the histological analysis of large cohorts of patient CRC specimens supported the human relevance of aPKCs as repressors of serrated tumorigenesis.^{13–15} Therefore, this mouse model offers a unique opportunity to study the kinetics of the mechanisms of spontaneous tumor initiation in an oncogenic-agnostic system.¹³ Mouse and human CRC tumors deficient in the aPKCs are characterized by the existence of cancer cells with a metaplastic-fetal phenotype (termed tumor fetal-metaplastic cells [TFMCs]) as well as a different clusterin (CLU)-expressing population of cancer cells with features reminiscent of the revival stem cells (RSCs) (termed tumor RSCs [TRSCs]) reportedly shown to arise upon intestinal damage.^{16,17} However, the impact that the loss of aPKCs has on the adult intestinal stem cell (ISC) compartment, as well as on the sequence of signaling mechanisms that control metaplasia, remains to be determined. Given that the accumulation of TFMCs correlates with the worst prognosis in CRC, identifying the signaling cascades whereby the loss of aPKCs drives the generation of this type of invasive cells is of paramount importance.

In this study, we have addressed how aPKCs regulate the ISC compartment and the metaplastic process during preneoplasia. Our results identify that JNK activation by aPKC deficiency in the intestinal epithelium regulates ISC homeostasis and that its cooperation with YAP is a critical event in promoting metaplasia.

RESULTS

aPKC deficiency drives stem cell loss and metaplasia in mouse-serrated tumors

To test the hypothesis that potential defects in the stem cell compartment could underlie the initial steps on tissue damage and metaplastic activation characteristic of aPKC-deficient intestinal tumors, we stained the intestine of *Prkci*^{fl/fl}*Prkcz*^{fl/fl}*Villin-Cre* mice with markers of ISCs (OLFM4), RSC (CLU), and fetal-metaplastic cells (TFMCs) (ANXA10 in the small intestine and MUC5AC in the colon). Tumors from these mice were completely devoid of OLFM4-expressing stem cells, which correlated with the presence of two other cell populations expressing CLU (TRSC) or ANXA10 (TFMC), respectively (Figure 1A). OPAL-multiplex immunofluorescence (OMI) demonstrated the absence of OLFM4⁺ cells and the mutually exclusive presence of TFMCs and TRSCs in *Prkci*^{fl/fl}*Prkcz*^{fl/fl}*Villin-Cre* tumors (Figure 1B). *In situ* hybridization (ISH) showed that LGR5⁺ cells were absent in *Prkci*^{fl/fl}*Prkcz*^{fl/fl}*Villin-Cre* tumors concomitant with the complete deletion of aPKCs (Figures 1C and S1A).

We next analyzed single-cell RNA sequencing (scRNA-seq) data of tumor and non-tumor areas from *Prkci*^{fl/fl}*Prkcz*^{fl/fl}*Villin-Cre* intestinal tissues integrated with data from wild-type (WT) mice (pool of samples from *Prkci*^{fl/fl}, *Prkcz*^{fl/fl}, and *Prkci*^{fl/fl}*Prkcz*^{fl/fl} mice). This analysis identified a robust reduction of ISC markers (*Olfm4*, *Lgr5*, *Ascl2*, and *Gkn3*) in *Prkci*^{fl/fl}*Prkcz*^{fl/fl}*Villin-Cre* tumors (Figures 1D–1F and S1B–S1F). Stem-related and stem-subsets signatures from a recent study defining a gradient of stemness

(ISC-I > ISC-II > ISC-III) in normal ISCs¹⁸ showed a clear reduction of these signatures in aPKC-deficient tumors (Figures 1G and 1H), which is not a consequence of tumorigenesis since a similar phenotype was observed in the non-tumor region (Figures 1D–1H and S1B–S1F). These initial observations suggested that serrated tumor initiation is not only associated with a strong metaplastic response but also with the loss of the ISC compartment.

Stem cell loss in human premalignant lesions and CRC associates with aPKC deficiency

We next investigated whether aPKC loss, its association with defects in the stem cell compartment, and the activation of the metaplastic programs are early events during tumor initiation in human CRC. The analysis of premalignant lesions in a large cohort of 54 colonic dysplastic adenomas (including 28 conventional and 23 serrated) from 35 human patients identified that aPKC expression was reduced in 100% of SAs and 71% of CAs (Figures 2A–2C and S2A–S2C). This suggests that human SAs cannot initiate in the context of high aPKC, but although CAs can exist despite high aPKC levels, the majority occur in aPKC-low conditions. OLFM4 was negative in 100% of SAs and 90% of the aPKC-deficient CAs (Figures 2B, 2C, and S2B). In this regard, 50% of the aPKC-proficient adenomas (all being conventional) are OLFM4-deficient, indicating that while SAs are aPKC/OLFM4 double-negative, approximately half of CAs are OLFM4-deficient tumors through a mechanism that is independent of aPKC deficiency (Figure S2B). Nonetheless, combining SA and CA, nearly 100% of all the aPKC-low cases are OLFM4-defective. These data indicate that OLFM4 tumor positivity is incompatible with aPKC deficiency, irrespective of the adenoma histological type. Also, of all the aPKC-low cases, about 67% are MUC5AC⁺, and only 10% of the aPKC-high lesions are MUC5AC⁺, indicating that metaplasia is under the strict control of aPKCs (Figures 2B, 2C, and S2C). These observations support the conclusion that aPKC deficiency, the ensuing loss of stem cells, and the resulting metaplastic response occur at an early step during tumorigenesis in human patients. The reduced OLFM4 levels in aPKC-deficient CAs were consistent with the analysis of mouse tumors in which APC was selectively deleted in the intestinal epithelial cells (IECs) with or without the compound deletion of both aPKCs. Like in human CAs, aPKC deficiency in mouse CAs resulted in the lack of OLFM4⁺ cells and the concomitant accumulation of ANXA10⁺ cells (Figure S2D).

To establish the relevance for advanced CRC, two large independent cohorts of surgically resected CRC specimens were analyzed by OMI. Among the 490 CRC patients in the tissue microarray (TMA), 438 with available clinical information were stratified according to PKC λ/ι and PKC ζ expression levels (Figures 2D–2F). Contingency analysis identified that CRC patients with high PKC λ/ι expression showed more OLFM4⁺ cases than those with low PKC λ/ι , irrespective of the levels of PKC ζ (Figures 2G and 2H). Consistently, multivariate logistic regression analysis demonstrated that high PKC λ/ι expression correlated with OLFM4⁺ cases independent of other pathological features (Figure S2E), indicating that PKC λ/ι plays a major role in maintaining the stem cell pool in CRC human tumors. In agreement with the phenotype of the mutant mice, most PKC λ/ι -low tumors are also PKC ζ -low (approximately 80%), whereas very few tumors that

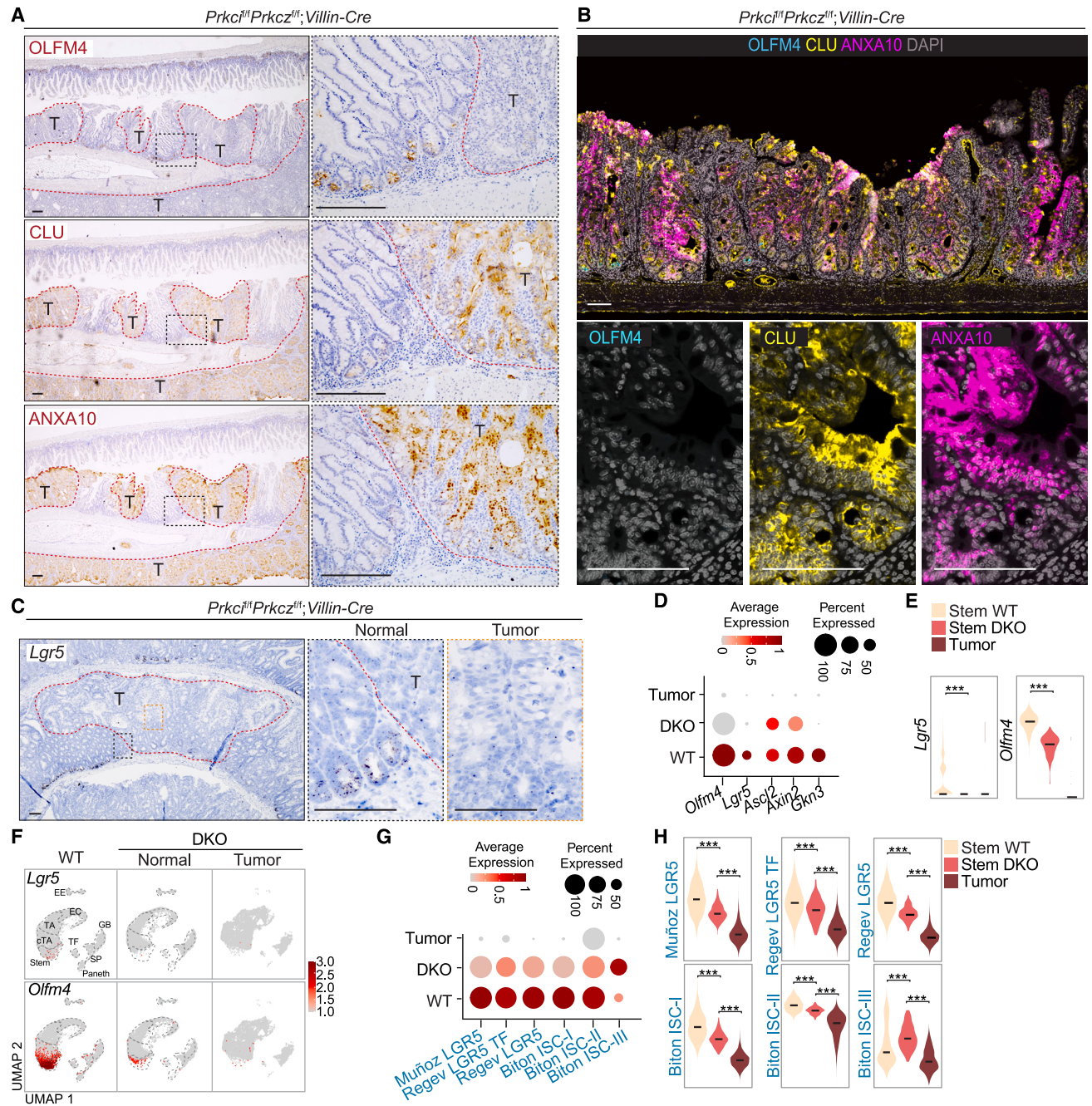


Figure 1. aPKC deficiency drives stem cell loss

(A) Immunohistochemistry (IHC) for OLFM4, CLU, and ANXA10 in *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-Cre* tumors.

(B) Immunofluorescence (IF) for OLFM4 (cyan), CLU (yellow), and ANXA10 (magenta) in non-tumor and tumoral regions of *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-Cre* mice.

(C) ISH for *Lgr5* in non-tumor and tumoral regions of *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-Cre* mice.

(D and E) Dotplot (D) and violin plot (E) of stem cell markers from scRNA-seq data of *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-Cre* tumors (Tumor) and non-tumoral stem cells of *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-Cre* (DKO) and *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-Cre* (WT) mice.

(F) Uniform manifold approximation and projection (UMAP) plots colored by *Lgr5* or *Olfr4* expression.

(G and H) Dotplot (G) and violin plot (H) of stem-related signatures.

Scale bars: 100 μ m in (A)–(C). Quantitative data (E) and (H) presented as median values (horizontal lines). p values were calculated by unpaired t test. *** $p < 0.001$. See also Figure S1.

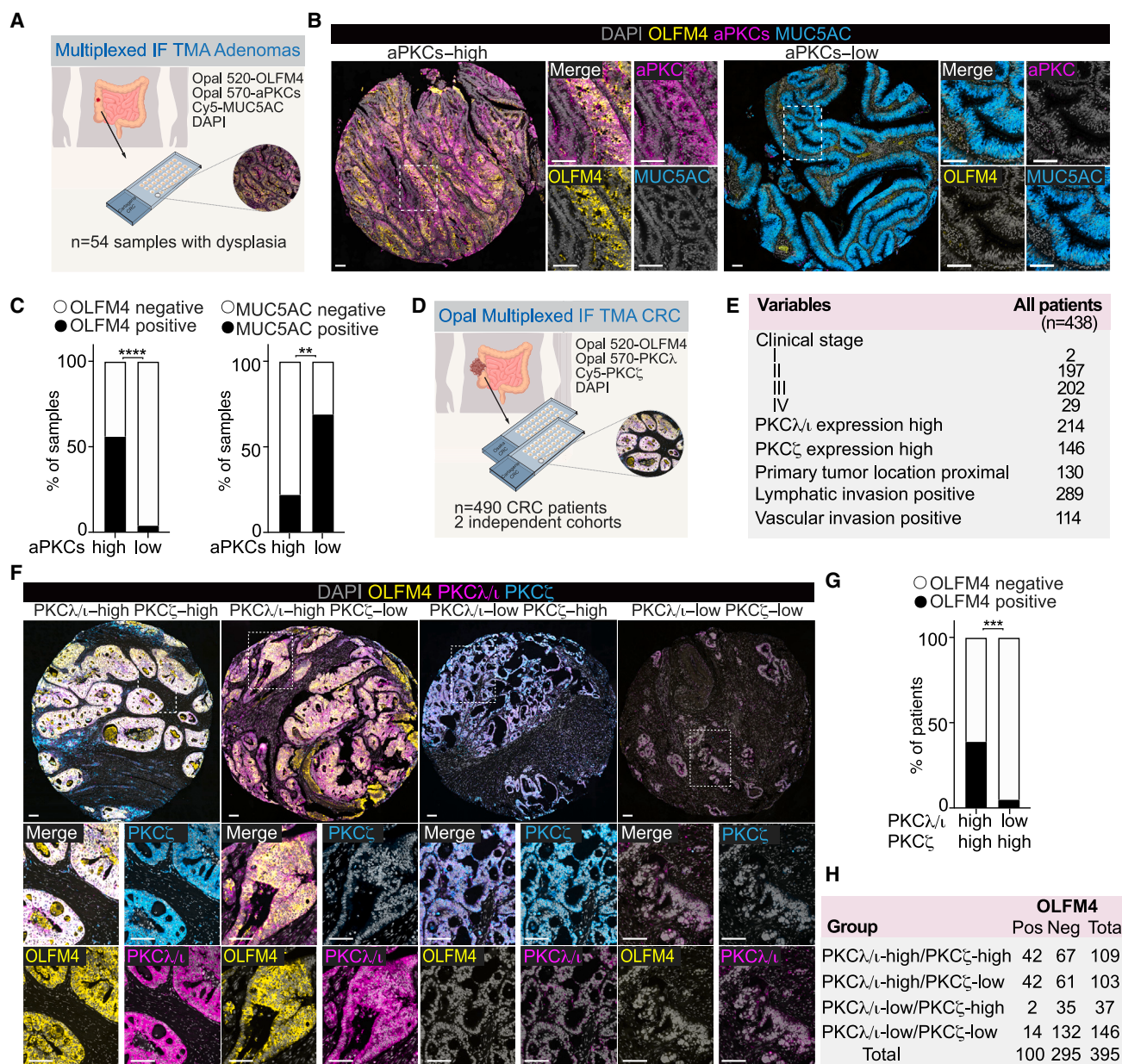


Figure 2. Low aPKC levels are associated with stem cell loss in human premalignant lesions and CRCs

(A–C) Analysis of colonic adenomas. Experimental design (A); IF for OLFM4 (yellow), aPKCs (magenta), and MUC5AC (cyan) (B); and contingency analyses of the correlation between expression of aPKCs and OLFM4 or MUC5AC (C).

(D–H) Analysis of colorectal cancer (CRC) samples. Experimental design (D); baseline clinical and pathological characteristics and the expression levels of PKCλ/ι and PKCζ (E); IF for OLFM4 (yellow), PKCλ/ι (magenta), and PKCζ (cyan) (F); contingency analysis of the correlation between expression of OLFM4 and PKCλ/ι within the PKCζ high cases (G); and expression of OLFM4 in the four groups classified by PKCλ/ι and PKCζ expression (H).

Scale bars: 100 μm in (B) and (F). Chi-squared test (C) and (G). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

See also Figure S2.

are PKCλ/ι-low are PKCζ-high (approximately 20%) (Figure 2H). These results are consistent with a dual role of PKCλ/ι deficiency, whereby it triggers a preneoplastic cascade that is thwarted by the simultaneous activation of a PKCζ-dependent interferon-driven anti-tumor CD8⁺ T cell response.¹⁹ Therefore, like in mice, both aPKCs must be simultaneously reduced for intestinal tumor development to proceed in humans.

PKCλ/ι critically regulates stem cell transcriptional programs

We next analyzed the non-tumor part of the intestinal epithelium of *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* mice compared with mice in which PKCλ/ι (*Prkci^{fl/fl};Villin-Cre*) or PKCζ (*Prkcz^{fl/fl};Villin-Cre*) were singly and selectively inactivated. We found a reduction in OLFM4⁺ and LGR5⁺ cells in non-tumor intestinal tissues, which were

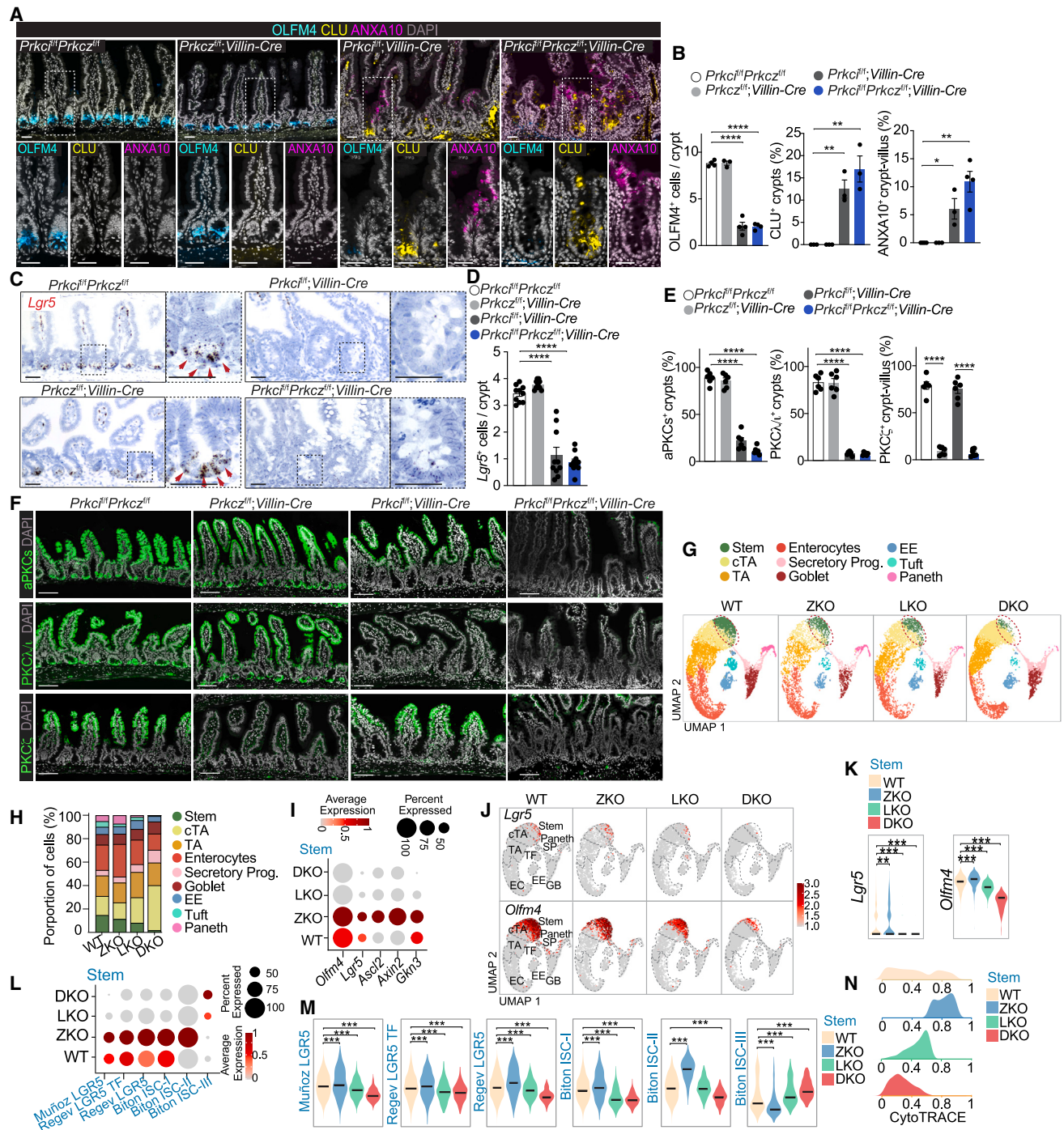


Figure 3. PKC λ/ι is a critical regulator of stem cell transcriptional programs

(A and B) IF for OLFM4 (cyan), CLU (yellow), and ANXA10 (magenta) in small intestine (A) and quantification (B).

(C and D) ISH for *Lgr5* in small intestine (C) and quantification (D).

(E and F) Staining of aPKC, PKC λ/ι , and PKC ζ in intestinal sections (F) and quantification (E).

(G) UMAP of epithelial cells colored by epithelial cell types in scRNA-seq of small intestinal epithelial cells (IECs) from WT, *Prkcz*^{fl/fl}; Villin-Cre (ZKO), *Prkci*^{fl/fl}; Villin-Cre (LKO), and *Prkci*^{fl/fl}*Prkcz*^{fl/fl}; Villin-Cre (DKO) mice. TA: transient amplifying; cTA: cycling TA; EE: enteroendocrine.

(H) Epithelial-cell-type percentage relative to total epithelial cells.

(I) Dotplot of stem cell markers in the stem cell compartment.

(J) UMAP plots colored by *Lgr5* or *Olfm4* expression.

(legend continued on next page)

substituted by CLU⁺ cells at the bottom of the crypts in not only *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* but also in *Prkci^{fl/fl};Villin-Cre* mice (Figures 3A–3C). These crypts showed a distorted morphology and hyperplastic appearance, with ANXA10 expression at the top positions (Figure 3A), consistent with the notion that the metaplastic response is a primary event preceding intestinal tumorigenesis. Immunofluorescence analysis using isotype-specific antibodies demonstrated the selective loss of each aPKC isoform in the corresponding knockout in the IECs (Figures 3E and 3F). CLU and MUC5AC were preferentially expressed in the non-tumor regions of the proximal but not distal colon (Figures S3A and S3B), in agreement with *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* mice showing a preference for developing tumors in that same colonic region, replicating the right-side characteristics of serrated neoplasia in humans.¹³ Moreover, although aPKC-deficient tumors rely on intestinal inflammation,¹³ treatment with a combination of broad-spectrum antibiotics to reduce microenvironmental inflammation did not revert the stem cell loss or the induction of metaplasia (Figures S3C–S3E), demonstrating that the impact of aPKC deficiency in intestinal homeostasis is intrinsic to their loss in the intestinal epithelium.

scRNA-seq data analysis of intestinal crypt-enriched epithelial cells from different genotypes identified nine major epithelial cell types (Figures 3G and S3F–S3J). Differences in the progenitor populations were found in the *Prkci^{fl/fl};Villin-Cre* mice, which were even more significant in *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* mice (Figures 3G and 3H). In keeping with previous observations,²⁰ we also found a significant increase in ISC markers and stem-related signatures in the PKCζ-deficient stem cells (Figures 3I–3M and S3K). By contrast, the remaining PKCλ/- and aPKC-deficient ISCs showed a robust reduction in stem transcriptional programs (Figures 3I–3M and S3K), indicating that PKCλ/- and PKCζ play antagonistic roles in stem cell homeostasis but that PKCλ/- deletion dominates the stem cell function when simultaneously inactivated with PKCζ. Consistently, CytoTRACE scores demonstrated that PKCλ/- and aPKC-deficient ISCs were skewed toward a low stem potential (Figure 3N). These results established that the loss of PKCλ/- in the intestinal epithelium, either alone or in combination with that of PKCζ, reduces the stem cell pool, which we posit is an integral part of the chronic damage-regeneration cycle underlying tumor initiation in serrated carcinogenesis.

Stem cell defects by aPKC inactivation precede epithelial cell metaplasia

To investigate the spatiotemporal relationship between aPKC loss, the defects in the ISC compartment, and the metaplastic response in the early steps of tumor initiation, we induced the short-term deletion of PKCλ/- or both aPKCs in IECs. To that end, we generated new mouse lines in which either *Prkci* or both aPKCs can be inducibly and acutely deleted by tamoxifen (TAM; *Prkci^{fl/fl};Villin-CreERT2* or *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-CreERT2*).

We found that the simultaneous deletion of both aPKCs gives a strong reduction in the stem cell population as early as day 3, remaining low at day 7 post-deletion (Figures 4A–4E). By contrast, CLU⁺ cells were undetected until day 4, and ANXA10⁺ cells were undetected until day 5 (Figures 4B and 4C). CLU⁺ cells appeared at the bottom of the crypts, while ANXA10⁺ cells localized at the crypt-top positions (Figure 4B), paralleling increased Ki67⁺ proliferative cells and crypt deformation (Figures S4C and S4D). Furthermore, qPCR analysis demonstrated the decrease in *Lgr5* and *Olfm4* transcripts and the increased expression of *Clu* and *Anxa10* concomitant with the efficient knockout of both aPKCs (Figure 4F). When the same type of experiment was carried out in *Prkci^{fl/fl};Villin-CreERT2* mice, we found that the single loss of PKCλ/- was sufficient to phenocopy the deletion of both aPKCs (Figures 4G–4L and S4E–S4G). These data demonstrated that losing LGR5⁺ stem cells in PKCλ/- or aPKC-deficient IECs precedes crypt damage and the induction of CLU⁺ RSCs, which are chronologically followed by the appearance of ANXA10⁺ FMCs.

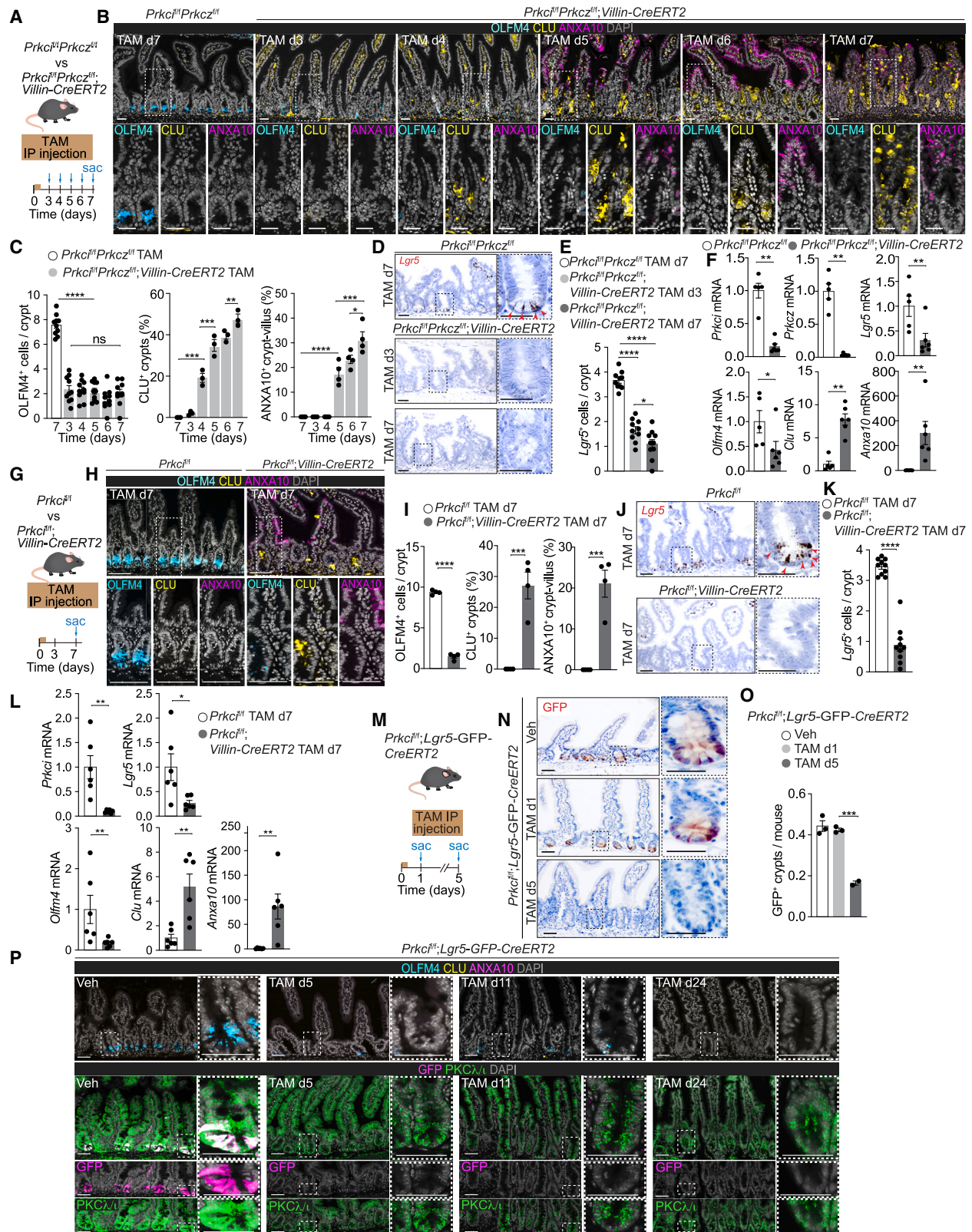
To determine the effect of *Prkci* deletion directly and selectively in LGR5⁺ ISCs, we generated *Prkci^{fl/fl};Lgr5-EGFP-IRES-CreERT2* mice. In this mouse line, LGR5⁺ cells are GFP labeled, and PKCλ/- is selectively deleted in GFP⁺ cells upon TAM treatment (Figure 4M). Histological evaluation showed an inducible reduction in the *Lgr5*-GFP⁺ stem cells upon *Prkci* deletion, concomitant with a reduction in GFP and OLFM4 expression (Figures 4N–4P), demonstrating that PKCλ/- is required to maintain the ISC population. Furthermore, we found reduced levels of enterocyte differentiation, as determined by alkaline phosphatase (ALP) staining, and abnormal differentiation of the secretory lineage with the accumulation of an intermediate immature goblet-cell-like phenotype, as shown by double LY2/AB staining (Figure S4H). These results are in agreement with previous data showing that enterocytes in intestinal crypts can dedifferentiate to replace lost LGR5⁺ stem cells.²¹ The observed defect in Paneth differentiation is fully consistent with our previous report demonstrating a similar phenotype upon deletion of PKCλ/- in the *Villin-Cre* model.²² Notably, the selective deletion of PKCλ/- in LGR5⁺ cells was insufficient to unleash the induction of the RSC or FMC populations, not even at day 24 post-induction (Figure 4P). These results demonstrated that the loss of ISCs and the subsequent induction of the revival-metaplastic response are two separate events controlled by the loss of aPKCs.

To further assess that the role of PKCλ/- in ISCs is functionally relevant for the regenerative potential of the intestinal epithelium *in vivo*, *Prkci^{fl/fl};Villin-Cre* and *Prkci^{fl/fl}* mice were subjected to 10 Gy of whole-body ionizing irradiation (Figure S4I). Whereas *Prkci^{fl/fl}* (WT) mice started to regain their body weight at day 5 post-irradiation, *Prkci^{fl/fl};Villin-Cre* mice never recovered, demonstrating an impairment in the regeneration of PKCλ/- deficient tissues after damage (Figure S4J). Histological evaluation

(K–N) Violin plot of *Lgr5* or *Olfm4* (K), dotplot of stem-related signatures (L), violin plots of stem-related signatures (M), and ridge plots of CytoTRACE score distributions (N) of stem cells.

Quantitative data are presented as mean ± SEM (B), (D), and (E) or median (K) and (M); horizontal lines. *n* = 10 (B), 10 (D), or 6 (E) fields. *p* values were calculated with one-way ANOVA and post hoc Tukey's test (B), (D), and (E) or unpaired *t* test (K) and (M). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. Scale bars: 50 μm in (A) and (C) and 100 μm in (F).

See also Figure S3.



(legend on next page)

showed that PKC λ /I-deficiency resulted, under these conditions, in massive scar areas, ruptures of the epithelial barrier, reduced Ki67⁺ cells, and increased TUNEL staining (Figures S4K–S4M). Consistent with a site preference in the damage of aPKC-deficient intestines, irradiation affected the proximal areas of the colon more than the distal ones (Figure S4N). Furthermore, the exposure of *Prkci*^{fl/fl}; *Lgr5-EGFP-IRES-CreERT2* mice to a similar irradiation protocol showed a drop in *Lgr5*-GFP⁺ stem cells associated with severe intestinal damage (Figures S4O–S4Q), consistent with a critical role for PKC λ /I in LGR5⁺ cell function and intestinal regeneration.

Cell-autonomous regulation of stem cell function by the aPKCs

Ex vivo experiments demonstrated that intestinal organoids from *Prkci*^{fl/fl}; *Villin-CreERT2* and *Prkci*^{fl/fl}; *Prkcz*^{fl/fl}; *Villin-CreERT2* mice responded poorly to WNT3A stimulation and failed to form viable organoids from single cells (Figures 5A–5F). Concomitant with the reduced expression of *Lgr5* and *Olfm4*, there was a robust expression of *Clu* and *Anxa10* (Figures 5G and 5H). Similar results were obtained when this experiment was carried out in colon organoids, which showed disintegration upon aPKC deletion, indicative of an abnormal stem cell function (Figures S5A–S5C). In agreement with these observations, gene set enrichment analysis (GSEA) of transcriptomic profiling of colonic organoids identified that the simultaneous deletion of PKC λ /I and PKC ζ resulted in a decrease in signatures related to LGR5⁺ stem cell function while increasing signatures associated with intestinal regeneration and metaplastic-fetal programs (Figures S5D–S5F). These analyses also consistently identified *Clu* as the most significantly upregulated gene along with *Lgr5* reduction (Figure S5G), identifying a profound cell-autonomous impairment of stem cell function and the activation of the regenerative and metaplastic signatures *in vitro*, both in the small intestine and in the colon.

PKC λ /I deletion in the intestinal epithelium drives AP-1 activation and cell death

To unravel the molecular mechanisms whereby the loss of aPKCs impairs ISC homeostasis and drives metaplasia, we investigated the chromatin accessibility landscape of IECs from mice of the four genotypes by genome-wide assay for transposase-accessible chromatin with sequencing (ATAC-seq). Hypergeometric optimization of motif enrichment (HOMER) analysis showed significant enrichment of AP-1 transcription factors (TFs) in the open chromatin regions of PKC λ /I- and

aPKC-deficient IECs (Figure S5H). Footprinting analysis also predicted increased activity in TFs with AP-1 motifs (Figure 5I). Interrogation of chromatin-accessible genomic areas showed increased binding of JUN and FOS as homo and heterodimers in PKC λ /I- and aPKC-deficient IECs (Figure S5I). Analysis of ATAC-seq, together with RNA-seq data, identified several AP-1-related TFs as the top regulators in PKC λ /I- and aPKC-deficient IECs (Figures 5J, 5K, S5J, and S5K). Enrichment analysis of genes with predicted binding of JUN family proteins as the top factors in ATAC-seq identified their potential role in regulating cell death in IECs (Figure 5L). Further analysis of our scRNA-seq data demonstrated that the remaining LGR5⁺ stem cells in *Prkci*^{fl/fl}; *Villin-Cre* and *Prkci*^{fl/fl}; *Prkcz*^{fl/fl}; *Villin-Cre* mice expressed high levels of *Jun* signatures (Figures 5M and 5N). Consistently, transcriptomic interrogation of colonic organoids identified that the simultaneous deletion of PKC λ /I and PKC ζ resulted in the activation of AP-1 and cell death transcriptional programs (Figure 5O).

JNK activation by aPKC deficiency is necessary for stem cell death and metaplasia

To investigate the mechanisms of cell death driven by aPKC deficiency in the LGR5⁺ population, we next determined JNK activity since this kinase system is an upstream regulator of the JUN pathway and cell death.^{23–25} Our data show that on day 1 after TAM injection in *Prkci*^{fl/fl}; *Lgr5-EGFP-IRES-CreERT2* mice, a time point before LGR5⁺ cells have been depleted, we found simultaneous staining for p-c-JUN and caspase-3 activity, and TUNEL in the GFP-labeled cell population (Figures 6A–6C). TAM-injected *Prkci*^{fl/fl}; *Villin-CreERT2* and *Prkci*^{fl/fl}; *Prkcz*^{fl/fl}; *Villin-CreERT2* mice showed that PKC λ /I- and aPKC-deficient crypts had massively increased p-c-JUN staining in both the small intestine and proximal colon (Figures 6D–6F and S6A–S6G). Interestingly, and consistent with the lack of activation of the revival-metaplastic responses in *Prkci*^{fl/fl}; *Lgr5-EGFP-IRES-CreERT2* mice (Figure 4P), cleaved caspase-3 or p-c-JUN was not activated upon deletion of PKC λ /I selectively in LGR5⁺ cells even 5 days after TAM injection (Figure S6H). These results support the conclusion that increased JNK activation by PKC λ /I deficiency causes ISC death and the independent activation of metaplasia in the non-ISC.

In keeping with the cell-intrinsic role of the aPKCs in ISC function, PKC λ /I deletion alone or combined with PKC ζ loss in organoids resulted in increased JNK and caspase-3 activity (Figures 6G and 6H). All these effects were accompanied by reduced organoid viability (Figures 6I, S6I, and S6J), which

Figure 4. Stem cell defects by aPKC inactivation precede epithelial cell metaplasia

(A–F) *Prkci*^{fl/fl}; *Prkcz*^{fl/fl} mice and *Prkci*^{fl/fl}; *Prkcz*^{fl/fl}; *Villin-CreERT2* mice treated with TAM, then analyzed after 3, 4, 5, 6, or 7 days. Experimental design (A); IF for OLFM4 (cyan), CLU (yellow), and ANXA10 (magenta) (B) and quantification (C); ISH for *Lgr5* (D) and quantification (E); qPCR in IECs from *Prkci*^{fl/fl}; *Prkcz*^{fl/fl} or *Prkci*^{fl/fl}; *Prkcz*^{fl/fl}; *Villin-CreERT2* mice treated with TAM, then analyzed after 7 days (F).

(G–L) *Prkci*^{fl/fl} and *Prkci*^{fl/fl}; *Villin-CreERT2* mice treated with TAM and analyzed after 7 days. Experimental design (G); IF for OLFM4 (cyan), CLU (yellow), and ANXA10 (magenta) (H) and quantification (I); ISH for *Lgr5* (J) and quantification (K); qPCR in IECs from *Prkci*^{fl/fl} and *Prkci*^{fl/fl}; *Villin-CreERT2* mice treated with TAM and analyzed after 7 days (L).

(M–P) *Prkci*^{fl/fl}; *Lgr5-EGFP-IRES-CreERT2* mice treated with vehicle or TAM and analyzed after 1, 5, 11, or 24 days. Experimental design (M); IHC for GFP (N); quantification of GFP⁺ crypts (O); IF for OLFM4 (cyan), CLU (yellow), ANXA10 (magenta; upper), PKC λ /I (green), and GFP (magenta; lower) (P).

Quantitative data are presented as mean $\pm$ SEM (C), (E), (F), (I), (K), (L), and (O). $n = 10$ (C, OLFM4), 3 (C, CLU), 4 (C, ANXA), 10 (E) and (K), or 4 (I) fields and 5 (F), 6 (L), or 3 (O) biological replicates. p values were calculated with one-way ANOVA and post hoc Tukey's test (C), (E), and (O) or unpaired t test (F), (I), (K), and (L).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars: 50 μ m in (B), (D), (H), (J), (N), and (P).

See also Figure S4.

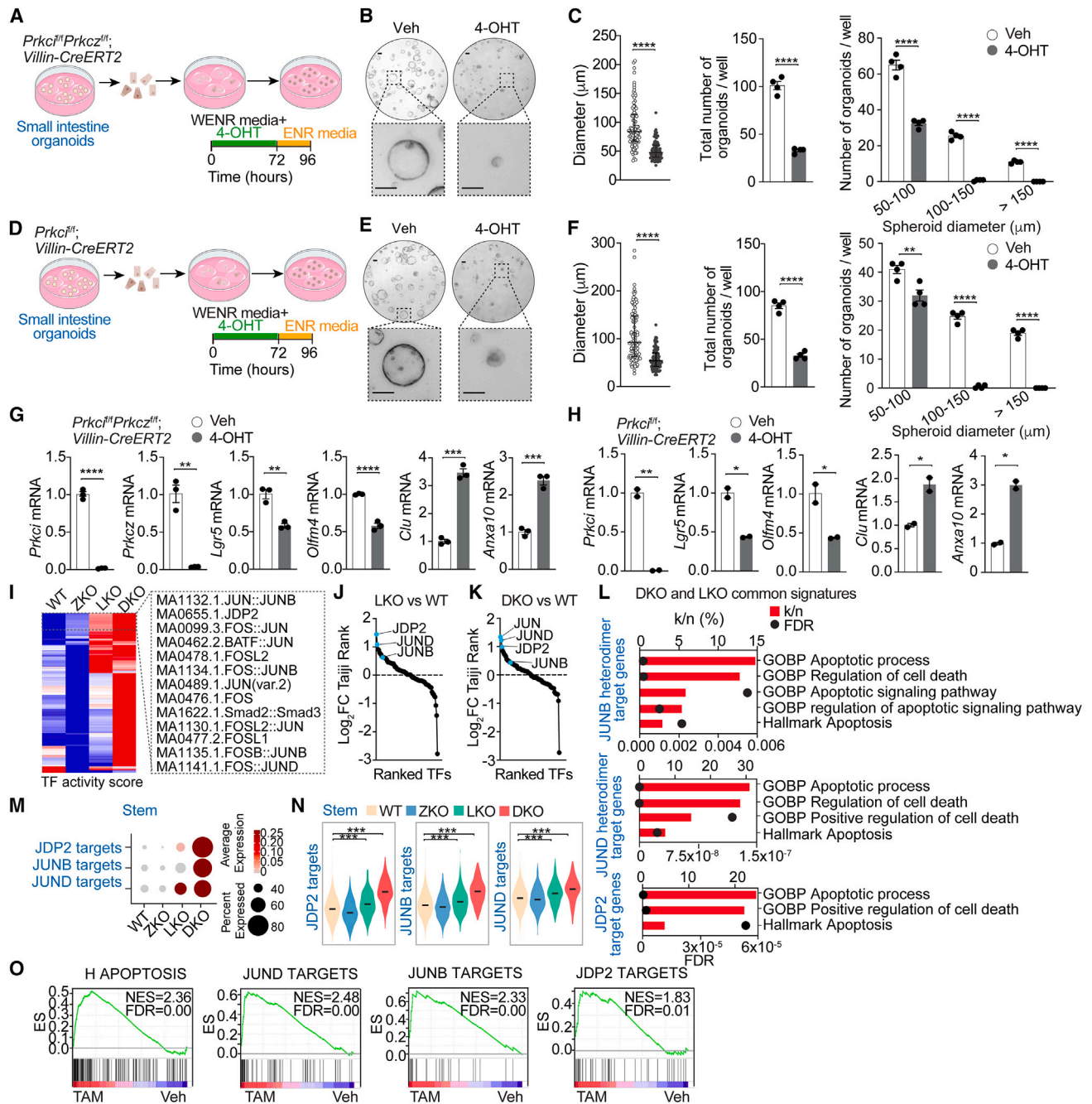


Figure 5. Cell-autonomous regulation of stem cell function by aPKCs

(A–C) Experimental design for 4-OHT induction of *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-CreERT2* organoids (A); representative pictures (B); and quantification of organoid formation and diameter (C).

(D–F) Experimental design for 4-OHT induction of *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-CreERT2* organoids (D); representative pictures (E); and quantification of organoid formation and diameter (F).

(G) qPCR in *Prkci^{fl/fl}Prkcz^{fl/fl}; Villin-CreERT2* organoids treated with or without 4-OHT for 4 days.

(H) qPCR in *Prkci^{fl/fl}; Villin-CreERT2* small intestine organoids treated with or without 4-OHT for 4 days.

(I) Heatmap of transcription factor (TF) activity score predicted by footprinting in the ATAC-seq of IECs.

(J and K) Differential PageRank score of TFs in LKO (J) or DKO (K) compared with WT IECs.

(L) Enrichment analysis of genes with predicted binding of JUND, JUNB, and JDP2.

(legend continued on next page)

was rescued by the pharmacological inhibition of cell death (Figures S6K and S6L), restoring the expression of *Lgr5* while enhancing that of *Anxa10* and having no effects on *Clu* (Figures 6J and 6K). These data suggest that whereas LGR5⁺ cells are exquisitely sensitive to cell death activation triggered by aPKC deficiency through JNK, revival and metaplastic cells can cope with increased cell death. In agreement with our hypothesis that enhanced JNK activation contributes to impaired crypt homeostasis in the aPKC-deficient intestinal epithelium, JNK pharmacological inhibition rescued the formation of viable organoids from single cells, as well as the decreased viability of aPKC-deficient organoids (Figures 6L–6P and S6M–S6O), and, like cell death blockade, also consistently rescued *Lgr5* to nearly normal levels (Figure 6Q). However, in contrast with the observation that cell death inhibition further enhanced *Anxa10* with no effects on *Clu*, JNK inhibition reduced the expression of both transcripts (compare Figures 6K and 6Q). Since deletion of PKC λ/ι has been shown to induce reactive oxygen species (ROS),²⁶ we tested whether blocking ROS production impaired JNK activation. Treatment with the ROS scavengers N-acetylcysteine (NAC) or butylated hydroxyanisole (BHA) inhibited the increased JNK activity in sgPrkci mouse IECs to levels similar to those of sgC cells, as measured by p-JNK and p-c-JUN immunoblotting (Figure S6P). These results indicate that PKC λ/ι loss unleashes JNK activation by a ROS-dependent mechanism.

PKC λ/ι depletion drives metaplasia through YAP/AP-1

Since *Anxa10* expression in aPKC-deficient organoids depends on JNK (Figure 6Q), we posited that increased AP-1 activity by PKC λ/ι deficiency might be a key regulator of metaplasia in this system. Consistent with this possibility, ATAC-seq data showed increased accessibility of the *Anxa10* promoter in PKC λ/ι - and aPKC-deficient IECs (Figure 7A). JASPAR interrogation identified several AP-1 (FOS and JUN) binding sites close to the *Anxa10* transcription start site that are adjacent to TEAD motifs (Figures 7A and S7A), suggesting that genomic co-occupancy might lead to cooperation between JNK/AP-1 and the YAP/TEAD pathways. Chromatin immunoprecipitation (ChIP)-PCR demonstrated the increased binding of JUN and TEAD to the ANXA10 promoter (Figure 7B). GSEA identified enriched YAP and AP-1 signatures in both aPKC-deficient colonic organoids and tumors (Figures 7C and 7D). Furthermore, PKC λ/ι deficiency increased the interaction of JUN and YAP with TEAD as determined by proximity ligation assay (Figures 7E and 7F). Consistently, JNK inhibition decreased *Anxa10* expression in aPKC-deficient organoids (Figure 6Q), and co-treatment with JNK and YAP inhibitors rescued *Anxa10* to nearly normal levels (Figures 7G and 7H). The importance of YAP activation under these conditions is underscored by the observation that treatment of organoids with the YAP inhibitor (Figures S7B–S7D) or the knockdown of YAP using short hairpin RNA (shRNA) (Figures S7E–S7H) reduced mouse and human organoid activity, selectively under conditions of aPKC deficiency. The inducible

deletion of the aPKCs promoted the simultaneous activation of p-c-JUN and YAP with a kinetic consistent with the metaplastic response (Figures 7I and 7J). Moreover, the analysis of non-transformed and tumoral tissues of *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* mice identified a robust overlap of p-c-JUN and YAP stainings in ANXA10⁺ cells (Figure 7K). Similar data were obtained in colonic tissues using MUC5AC as the metaplastic marker (Figure S7I). YAP and AP-1 signatures were also applied to the epithelial compartment of our previously published scRNA-seq analysis of *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* tumors,¹⁷ clustered in three tumor populations (Figure 7L). These include tumor cycling transient amplifying (TcTA), TRSC, and TFMC. This analysis showed significant enrichment of YAP and AP-1 signatures in TFMC (Figures 7L–7O), with a substantial overlap of both signatures in this tumoral population (Figure 7O). Consistently, TFMC accounts for 91% of AP-1/YAP double-positive cells (Figure 7P). These results support the notion that both AP-1 and YAP pathways are expressed in the same cell population undergoing the metaplastic lineage transition.

Low aPKC levels correlate with YAP/AP-1 activity in human patients

In keeping with the importance and human relevance of our observations from mouse organoids and tissues, the AP-1 and YAP signatures were highly expressed in the TFMC compartment in two human CRC scRNA-seq datasets (Figures S7J–S7O). Also, the classification of the tumor epithelial cell compartment according to AP-1 and YAP expression in these patients showed a clear overlap of both signatures in tumor cells expressing the metaplastic program (Figures S7P–S7S), mirroring the findings in the *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* mouse model. Consistent with previous observations on the aggressive features of TFMCs,¹⁷ AP-1 and YAP pathways were upregulated in the poor-prognosis iCMS3 CRC subtype, as well as in right-side and T4-stage tumors (Figure S7T). Interrogation of the CRC dataset in The Cancer Genome Atlas (TCGA) demonstrated that the AP-1 signature derived from our mouse model highly correlated with YAP activation in primary CRC tumors (Figure S7U). TCGA patients with low aPKC levels displayed enriched AP-1 and YAP signatures in the metaplastic-related poor-prognosis iCMS3 subtype (Figure S7V). These results support a molecular link between the fetal/metaplastic state and the synergistic activation of AP-1/YAP pathways driven by aPKC deficiency in human CRC. Because our data demonstrated that aPKC levels are reduced in all SAs and a significant proportion of CAs (Figures 2B and S2A), we next determined whether the activation of the AP-1 and YAP pathways was also an early event during tumor initiation. Indeed, simultaneous expression of p-c-JUN and YAP correlated with low aPKC levels and enhanced MUC5AC staining in the human premalignant cohort (Figures 7Q and S7W–S7Z). These results demonstrate that the activation of the AP-1/YAP pathways driven by the reduced levels of aPKC is an important event in CRC and early premalignant lesions, not only in mice but also in human patients.

(M and N) Dotplot (M) and violin plots (N) of JDP2, JUNB, and JUND targets in stem cells.

(O) GSEA plots of RNA-seq from colon organoids from *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-CreERT2* mice with or without 4-OHT.

Quantitative data are presented as the mean $\pm$ SEM (C), (F), (G), and (H) or median (N, horizontal lines) calculated by unpaired t test. $n = 100$ –200 (C) and (F), diameters) and $n = 4$ (C) and (F), 3 (G) and (O), or 2 (H) biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars: 100 μ m in (B) and (E). See also Figure S5.

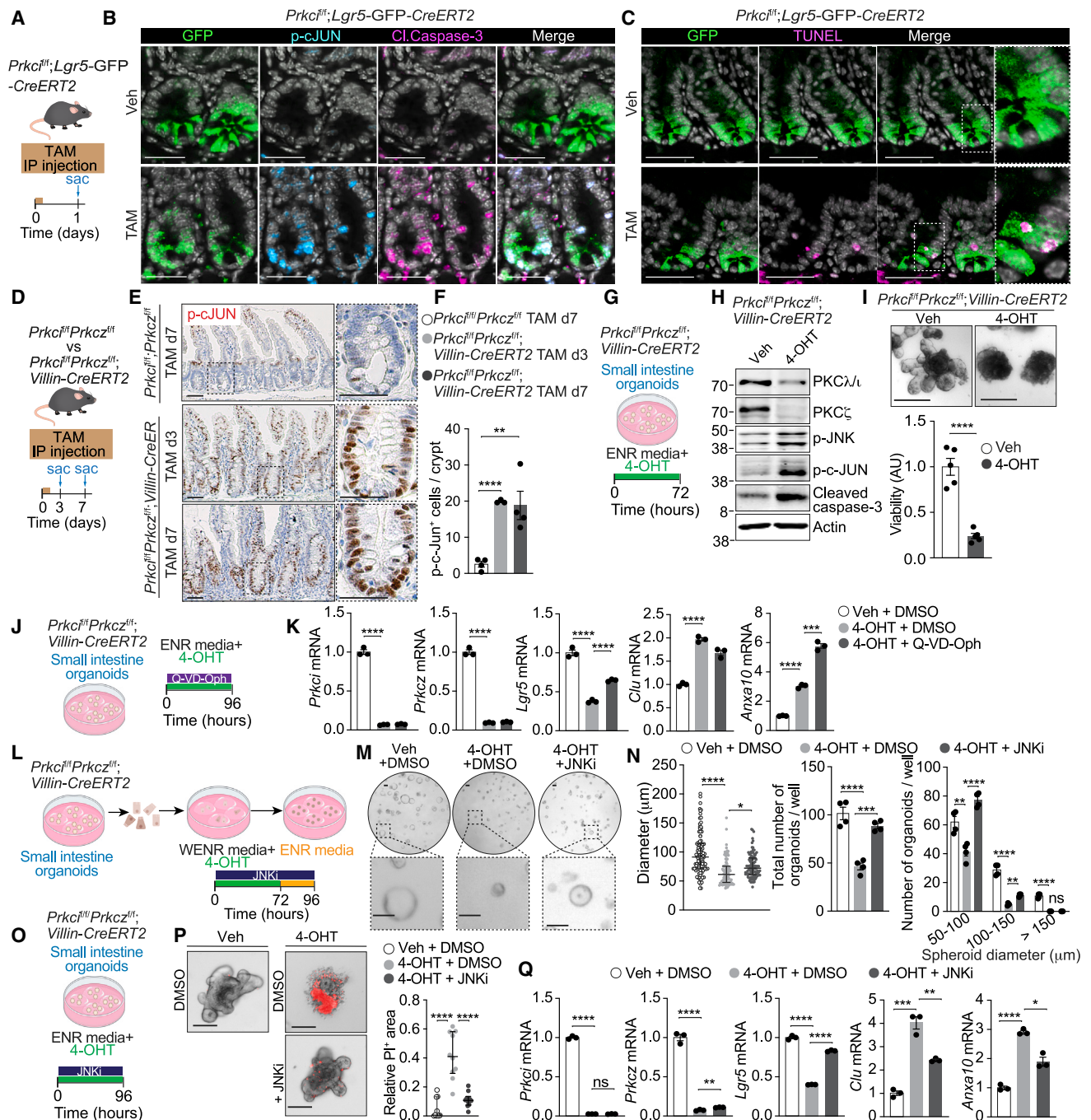


Figure 6. JNK activation by aPKC deficiency is necessary for stem cell death and metaplasia

(A–C) *Prkc1^{fl/fl};Lgr5-EGFP-IRES-CreERT2* mice treated with TAM and analyzed after 1 day. Experimental design (A); IF for GFP (green), p-cJUN (cyan), and cleaved caspase-3 (magenta) (B); and IF for GFP (green) with TUNEL staining (magenta) (C).

(D–F) *Prkc1^{fl/fl};Prkc2^{fl/fl}* and *Prkc1^{fl/fl};Prkc2^{fl/fl};Villin-CreERT2* mice treated with TAM and analyzed after 3 or 7 days. Experimental design (D), IHC for p-cJUN (E), and quantification (F).

(G–I) *Prkc1^{fl/fl};Prkc2^{fl/fl};Villin-CreERT2* small intestinal organoids treated with or without 4-OHT for 3 days. Experimental design (G); immunoblots (H) and representative bright-field pictures (top) and viability assay (bottom) (I).

(J and K) *Prkc1^{fl/fl};Prkc2^{fl/fl};Villin-CreERT2* organoids were treated with vehicle, 4-OHT, or 4-OHT combined with 30 μ M Q-VD-Oph for 4 days. Experimental design (J) and qPCR (K).

(legend continued on next page)

DISCUSSION

Our understanding of the molecular mechanisms controlling preneoplastic lesions and tumor initiation remains limited. Intestinal tumorigenesis has long been considered a paradigmatic example of a carcinogenic process driven by the spontaneous transformation of an adult cancer stem cell population due to the loss of the APC tumor suppressor and the consequent activation of the WNT pathway.² However, how that model fits with other more complex situations like those emerging through the serrated (APC-independent) pathway still needs to be fully understood.¹¹ In contrast with the initiation through the conventional pathway, the generation of serrated tumors involves a process of initial metaplastic transdifferentiation of uncertain origin.¹¹ Therefore, it has been proposed that transformation through the serrated pathway is dominated primarily by changes in the most differentiated cells residing at the top of the crypt, which must undergo a metaplastic switch to endure tissue damage, along with the gain of fetal features that confer the plasticity permissive for cancer transformation. Our study demonstrates that in addition to fetal-metaplastic transdifferentiation events, the earliest detectable change in serrated preneoplastic lesions is the loss of the stem cell population through the activation of cell death. This impairment in the crypt stem cell compartment is associated with the generation of the RSC compartment in close proximity to the disappearing ISCs, followed by a clearly defined spatiotemporal metaplastic switch in the more differentiated areas of the crypt-villus axis. We show here that although the inactivation of PKC λ/ι in the LGR5 population is sufficient for ISC death, the induction of the metaplastic response requires the additional loss of this kinase in the transient amplifying (TA)-enterocyte compartment, and is a cell-intrinsic property of the differentiated components of the crypt-villi since treating mice with antibiotics does not impair the generation of FMCs. The interrogation of a cohort of human preneoplastic specimens supports the relevance of these findings. We found that aPKC levels are reduced in all the dysplastic serrated lesions and at least 70% of CAs, which perfectly correlates with the lack of stem cells and metaplasia activation. Therefore, the role of aPKC in repressing the initial steps of tumorigenesis expands beyond the serrated pathway and is not a late event associated with or secondary to the acquisition of more aggressive features, but that is a very early event that might determine the course of the disease from the very beginning.

The impairment in the ISC function by aPKC deficiency should be considered in the bidirectional flux between stem cells (ISC, CBC) and RSC in intestinal injury and cancer.^{27,28} According to previous studies, this binary dynamic pool underlies the mechanism that results in the generation of a still poorly defined revival-fetal-gastric cell compartment.^{11,16,27,29–31} Our study goes

beyond the pioneering reports from other investigators on the initial characterization of the response to the loss of LGR5⁺ cells and establishes a bifurcation in this process. Thus, whereas high YAP and low WNT activity drive the fetal-metaplastic cluster, the RSC compartment still maintains relatively high levels of WNT activity but with moderate YAP activation and is no part of the metaplastic transdifferentiation process but rather creates a reservoir for rapid reconstitution of the LGR5⁺ pool upon damage recovery. Therefore, intestinal metaplasia, although coordinated with the loss of stem cells, is a separate mechanism from the ISC-RSC axis as it involves different transcriptional features and a distinct spatiotemporal evolution from that of the RSC. Thus, our results propose a model whereby both the FMCs and RSCs bifurcate depending on their WNT vs. YAP activity load, with the metaplastic cells becoming the ones with the most aggressive features when they undergo cancer transformation as demonstrated in our previously reported results.¹⁷ Thus, the accumulation of TFMCs correlates with poor survival in CRC patients and with the most aggressive iCMS3 phenotype, whereas TRSCs correlate with iCMS2 features, consistent with their WNT-enriched characteristics.¹⁷

We show here that the very early mechanism that initiates this process is the activation of JNK by aPKC deficiency, especially that of PKC λ/ι . JNK activation accounts for ISC death but also results in the triggering of an AP-1 cascade that cooperates with YAP to drive the metaplastic response, as evidenced by the existence of AP-1/TEAD open regions in metaplastic genes such as *Anxa10*, as well as their recruitment to the *ANXA10* promoter and their physical interaction. The sustained acquisition of the gastric-fetal metaplastic features by the cells along the crypt-villus axis through the JNK/AP-1/YAP cascade perpetuates this stem-cell-loss/metaplasia scenario that eventually culminates in serrated lesions and CRC, as well as in a significant proportion of CAs that present with reduced aPKC levels, ISC loss, and the FMC lineage switch. Although it is well-documented that metaplastic processes are often associated with chronic environmental damage and inflammation in which the breakdown of the intestinal epithelial barrier and the microbiota play key roles,³² our study provides compelling evidence that the activation of the intestinal metaplastic programs does not depend on microenvironmental inflammation as it can be recapitulated cell-autonomously in intestinal and colon organoids after the rapid inactivation of the aPKCs in the TAM-inducible knockout system. Therefore, although microenvironmental and desmoplastic components might also impinge in the process of metaplastic transformation, especially during the evolution to cancer, the initial trigger is the ability of aPKC-deficient IECs to activate the JNK/AP-1 cascade in a cell-autonomous manner, even before the first signs of carcinogenesis are detected.

(L–N) *Prkci*^{fl/fl}*Prkcz*^{fl/fl}*Villin-CreERT2* organoids were dissociated into single cells and cultured in WENR media with vehicle, 4-OHT or 4-OHT combined with JNK inhibitor (1 μ M JNK-IN-8) for the organoid formation assay. Experimental design (L); representative pictures (M); and quantification of organoid formation and diameter (N).

(O–Q) *Prkci*^{fl/fl}*Prkcz*^{fl/fl}*Villin-CreERT2* organoids were treated with vehicle, 4-OHT, or 4-OHT combined with JNK inhibitor (1 μ M JNK-IN-8) for 4 days. Experimental design (O); representative pictures and quantification of propidium iodide (PI) staining (P), and qPCR (Q).

Quantitative data (F), (I), (K), (N), (P), and (Q) are presented as mean $\pm$ SEM. $n = 4$ (B) and (C) mice, 3–4 (F) fields, 5 (I), 3 (K) and (Q), 4 (N, organoids) biological replicates, or 100 organoids (N, diameter). p values were calculated with one-way ANOVA and post hoc Tukey's test (F), (K), (P), and (Q) or unpaired t test (I) and (N). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars: 50 μ m in (B), (C), and (E) and 100 μ m in (I), (M), and (P).

See also Figure S6.

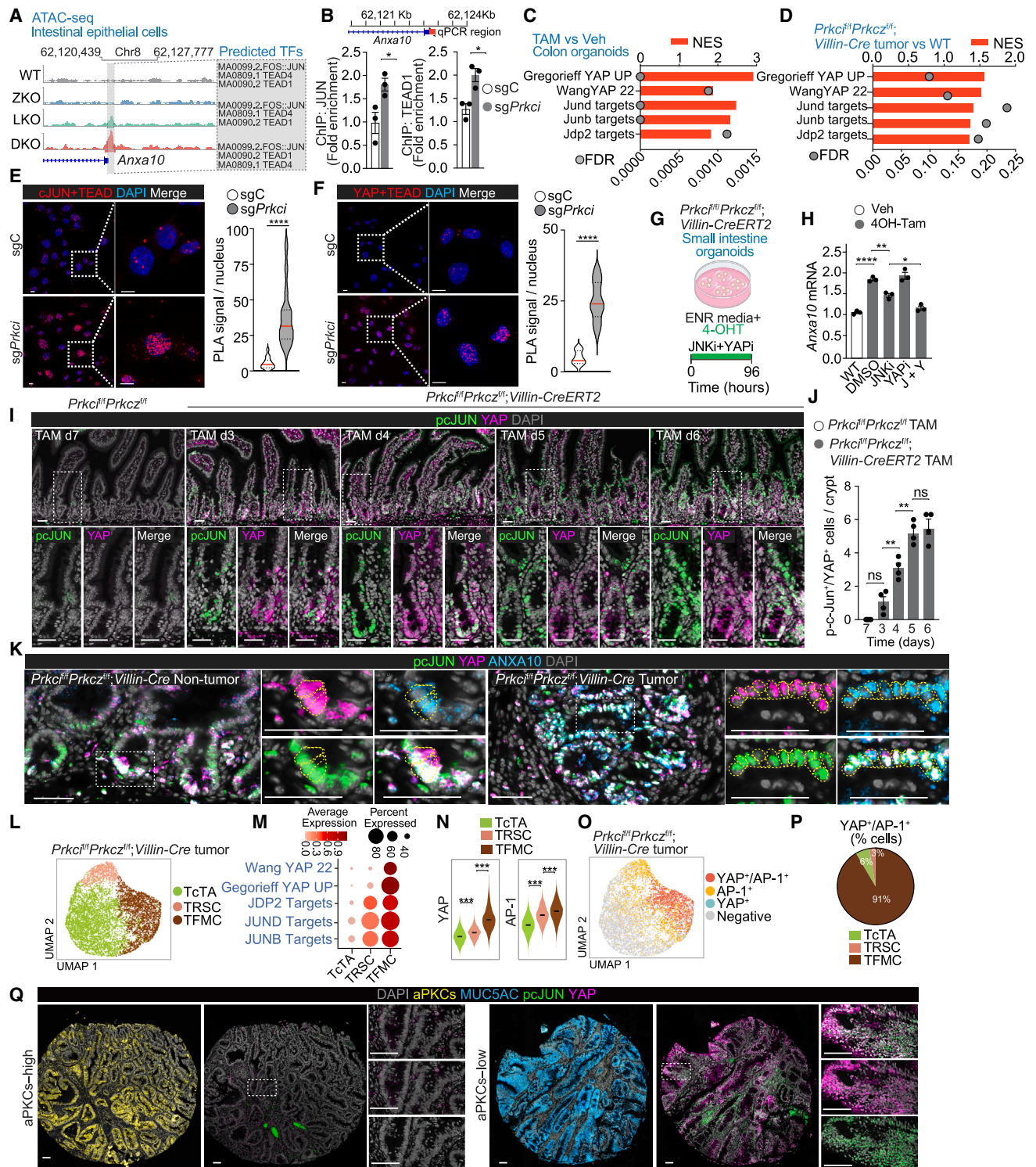


Figure 7. PKC λ /I depletion drives metaplasia through AP-1/YAP

(A) Genome browser view at *Anxa10* promoter in the ATAC-seq data of IECs with predicted TFs by JASPAR.

(B) ChIP-qPCR analysis of JUN and TEAD1 TFs for *Anxa10* promoter in sgC and sgPrkci small IECs.

(C and D) GSEA of YAP, JUNB, or JDP2 targets in *Prkci*^{fl/fl}*Prkcz*^{fl/fl}; Villin-CreERT2 colon organoids with or without 4-OHT treatment (C) or in tumors of *Prkci*^{fl/fl}*Prkcz*^{fl/fl}; Villin-Cre mice compared with WT tissue (D).

(legend continued on next page)

In summary, we demonstrate here that the activation of the revival-metaplastic processes triggered by the inactivation of the aPKCs in the intestinal epithelium is sequential, topologically separated, and is preceded by the loss of ISCs due to cell death triggered by JNK. Although spatially separated, the FMC and RSC compartments share a previously unappreciated activation of the JNK/AP-1 pathway. The enrichment in YAP in the FMC, as compared with a relatively sustained activation of the WNT pathway in the RSC compartment, underlies their spatial-temporal segregation during the initial response, which is contemporary to the loss of the ISC compartment, all triggered by aPKC inactivation. We propose that this metaplastic lineage change serves to help cells endure an inflammatory and oxidative hostile environment, which, when perpetuated, facilitates their oncogenic transformation to become cancer cells while still maintaining their initial phenotypic and lineage memory that makes them the aggressive cell pool in intestinal cancer, irrespective of their conventional or serrated origin.

Limitations of the study

Although our study demonstrates that the loss of the aPKCs is critical for the activation of the metaplastic programs preceding tumorigenesis, and the accumulation of these cells in CRC correlates with poorer prognosis, whether their ablation results in reduced malignancy will still need to be addressed in additional studies using genetic loss-of-function models. Further tracing experiments using reporter mice with deficiency in the aPKC genes will help establish the mechanisms whereby metaplasia predetermines the aggressiveness of CRC.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
 - Lead contact
 - Materials availability
 - Data and code availability
- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
 - Mice
 - Human samples
- **METHOD DETAILS**
 - Intestinal epithelial cell isolation and intestinal organoid culture

- Histology, Immunohistochemistry, Immunofluorescence, and in situ hybridization (ISH)
- In situ proximity ligation assay
- Immunoblotting Analysis
- RNA Extraction and Analysis
- Chromatin Immunoprecipitation (ChIP)
- ATAC-seq library preparation and sequencing
- 10x Library Preparation and Sequencing
- Analysis of scRNA sequencing data
- CytoTRACE Analysis
- Bulk RNA-seq and ATAC-seq analysis
- Tissue microarray analysis

● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.devcel.2024.05.001>.

ACKNOWLEDGMENTS

Research was supported by grants by the National Cancer Institute of the National Institutes of Health under awards numbers: R01CA265892, R01CA250025, and R01CA275846 to J.M.; R01CA246765 to M.T.D.-M.; R50CA265332 to A.D.; and R50CA283476 to J.F.L. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Project support for this research was provided in part by the European Union Horizon's 2020 grant (REVERT project GA848098) and the Center for Translational Pathology in the Department of Pathology and Laboratory Medicine, Weill Cornell Medicine. We thank Tavonna Bryant and Tararin Nikomborirak, the Epigenomics Core and the personnel of the Optical Microscopy Core at Weill Cornell Medicine, and Eric Rosiek of the Molecular Cytology Core at Memorial Sloan Kettering Cancer Center for technical assistance. J.M. and M.T.D.-M. are Homer T. Hirst III Professors of Oncology in Pathology.

AUTHOR CONTRIBUTIONS

Conceptualization, J.M., M.T.D.-M., H. Kinoshita, and A.M.-O.; methodology, J.M., M.T.D.-M., H. Kinoshita, and A.M.-O.; investigation, A.M.-O., H. Kinoshita, T.C.-D., Q.H., A.D., Y.M., X.Z., J.F.L., Y.N., and H. Kasashima; resources, H. Kasashima, M.Y., K.M., A.A.-G., D.T.-M., J.G.-S., P.C.-Z., and G.I.; writing – original draft, J.M., M.T.D.-M., H. Kinoshita, and A.M.-O.; writing – review & editing, all authors; supervision, J.M. and M.T.D.-M.; funding acquisition, J.M. and M.T.D.-M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

(E and F) Proximity ligation assay detection of endogenous cJUN/TEAD (E) and YAP/TEAD (F) interactions in sgC and sgPrkci small IECs. Nuclei were counterstained with DAPI (blue). The detected interactions are identified by fluorescent dots (red).

(G and H) Experimental design (G) and qPCR from *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-CreERT2* small intestinal organoids treated with 4-OHT combined with inhibitors of JNK (0.5 μ M JNK-IN-8) and/or YAP (2.5 μ M verteporfin) for 4 days (H).

(I and J) *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-CreERT2* mice treated with TAM, then analyzed after 3, 4, 5, 6, and 7 days. IF for p-c-JUN (green) and YAP (magenta) (I) and quantification (J).

(K) IF for p-c-JUN (green), YAP (magenta), and ANXA10 (cyan) in the tumor and non-tumor tissues in the small intestine of *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* mice.

(L) UMAP of tumor cells in *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* mice colored by the tumor cell types.

(M and N) Dotplot (M) and violin plots (N) of the YAP- or AP-1-related signatures in each tumor cell type from (L).

(O) UMAP of tumor cells in *Prkci^{fl/fl}Prkcz^{fl/fl};Villin-Cre* mice colored by the levels of the YAP- and AP-1-related signatures.

(P) Proportions of tumor cell types in all the cells positive for both YAP and AP-1 signatures from (L).

(Q) IF for aPKCs (yellow) with MUC5AC (cyan), or p-c-JUN (green) with YAP (magenta), in human preneoplastic lesions.

Quantitative data are presented as mean $\pm$ SEM (B), (H), and (J) or median (E), (F), and (N). $n = 3$ (B) and (H) biological replicates, 16 (E), 21 (F) cells, or 4 (J) fields. p values were calculated using unpaired t test (B), (E), (F), and (N) or one-way ANOVA and post hoc Tukey's test (J). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars: 10 μ m in (E) and (F), 50 μ m in (I), and 100 μ m in (K) and (Q).

See also Figure S7.

Received: August 1, 2023
Revised: December 19, 2023
Accepted: May 3, 2024
Published: May 29, 2024

REFERENCES

- Siegel, R.L., Wagle, N.S., Cercek, A., Smith, R.A., and Jemal, A. (2023). Colorectal cancer statistics, 2023. *CA Cancer J. Clin.* 73, 233–254. <https://doi.org/10.3322/caac.21772>.
- Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* 61, 759–767. [https://doi.org/10.1016/0092-8674\(90\)90186-i](https://doi.org/10.1016/0092-8674(90)90186-i).
- IJsspeert, J.E.G., Vermeulen, L., Meijer, G.A., and Dekker, E. (2015). Serrated neoplasia-role in colorectal carcinogenesis and clinical implications. *Nat. Rev. Gastroenterol. Hepatol.* 12, 401–409. <https://doi.org/10.1038/nrgastro.2015.73>.
- De Sousa E Melo, F., Wang, X., Jansen, M., Fessler, E., Trinh, A., de Rooij, L.P.M.H., de Jong, J.H., de Boer, O.J., van Leersum, R., Bijlsma, M.F., et al. (2013). Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat. Med.* 19, 614–618. <https://doi.org/10.1038/nm.3174>.
- Guinney, J., Dienstmann, R., Wang, X., de Reyniès, A., Schlicker, A., Soneson, C., Marisa, L., Roepman, P., Nyamundanda, G., Angelino, P., et al. (2015). The consensus molecular subtypes of colorectal cancer. *Nat. Med.* 21, 1350–1356. <https://doi.org/10.1038/nm.3967>.
- Crockett, S.D., and Nagtegaal, I.D. (2019). Terminology, molecular features, epidemiology, and management of serrated colorectal neoplasia. *Gastroenterology* 157, 949–966.e4. <https://doi.org/10.1053/j.gastro.2019.06.041>.
- Markowitz, S.D., and Bertagnoli, M.M. (2009). Molecular origins of cancer: molecular basis of colorectal cancer. *N. Engl. J. Med.* 361, 2449–2460. <https://doi.org/10.1056/NEJMra0804588>.
- Bennecke, M., Kriegl, L., Bajbouj, M., Retzlaff, K., Robine, S., Jung, A., Arkan, M.C., Kirchner, T., and Greten, F.R. (2010). Ink4a/Arf and onco-gene-induced senescence prevent tumor progression during alternative colorectal tumorigenesis. *Cancer Cell* 18, 135–146. <https://doi.org/10.1016/j.ccr.2010.06.013>.
- Rad, R., Cadiñanos, J., Rad, L., Varela, I., Strong, A., Kriegl, L., Constantino-Casas, F., Eser, S., Hieber, M., Seidler, B., et al. (2013). A genetic progression model of Braf(V600E)-induced intestinal tumorigenesis reveals targets for therapeutic intervention. *Cancer Cell* 24, 15–29. <https://doi.org/10.1016/j.ccr.2013.05.014>.
- Jackstadt, R., van Hooff, S.R., Leach, J.D., Cortes-Lavaud, X., Lohuis, J.O., Ridgway, R.A., Wouters, V.M., Roper, J., Kendall, T.J., Roxburgh, C.S., et al. (2019). Epithelial NOTCH signaling rewires the tumor microenvironment of colorectal cancer to drive poor-prognosis subtypes and metastasis. *Cancer Cell* 36, 319–336.e7. <https://doi.org/10.1016/j.ccell.2019.08.003>.
- Chen, B., Scurrah, C.R., McKinley, E.T., Simmons, A.J., Ramirez-Solano, M.A., Zhu, X., Markham, N.O., Heiser, C.N., Vega, P.N., Rolong, A., et al. (2021). Differential pre-malignant programs and microenvironment chart distinct paths to malignancy in human colorectal polyps. *Cell* 184, 6262–6280.e26. <https://doi.org/10.1016/j.cell.2021.11.031>.
- Joanito, I., Wirapati, P., Zhao, N., Nawaz, Z., Yeo, G., Lee, F., Eng, C.L.P., Macalino, D.C., Kahraman, M., Srinivasan, H., et al. (2022). Single-cell and bulk transcriptome sequencing identifies two epithelial tumor cell states and refines the consensus molecular classification of colorectal cancer. *Nat. Genet.* 54, 963–975. <https://doi.org/10.1038/s41588-022-01100-4>.
- Nakanishi, Y., Duran, A., L'Hermitte, A., Shelton, P.M., Nakanishi, N., Reina-Campos, M., Huang, F., Soldevila, F., Baaten, B.J.G., Tauriello, D.V.F., et al. (2018). Simultaneous loss of both atypical protein kinase C genes in the intestinal epithelium drives serrated intestinal cancer by im-pairing immunosurveillance. *Immunity* 49, 1132–1147.e7. <https://doi.org/10.1016/j.immuni.2018.09.013>.
- Reina-Campos, M., Diaz-Meco, M.T., and Moscat, J. (2019). The dual roles of the atypical protein kinase Cs in cancer. *Cancer Cell* 36, 218–235. <https://doi.org/10.1016/j.ccell.2019.07.010>.
- Nakanishi, Y., Diaz-Meco, M.T., and Moscat, J. (2019). Serrated colorectal cancer: the road less travelled? *Trends Cancer* 5, 742–754. <https://doi.org/10.1016/j.trecan.2019.09.004>.
- Ayyaz, A., Kumar, S., Sangiorgi, B., Ghoshal, B., Gosio, J., Ouladan, S., Fink, M., Barutcu, S., Trcka, D., Shen, J., et al. (2019). Single-cell transcriptomes of the regenerating intestine reveal a revival stem cell. *Nature* 569, 121–125. <https://doi.org/10.1038/s41586-019-1154-y>.
- Martinez-Ordoñez, A., Duran, A., Ruiz-Martinez, M., Cid-Diaz, T., Zhang, X., Han, Q., Kinoshita, H., Muta, Y., Linares, J.F., Kasashima, H., et al. (2023). Hyaluronan driven by epithelial aPKC deficiency remodels the microenvironment and creates a vulnerability in mesenchymal colorectal cancer. *Cancer Cell* 41, 252–271.e9. <https://doi.org/10.1016/j.ccell.2022.11.016>.
- Biton, M., Haber, A.L., Rogel, N., Burgin, G., Beyaz, S., Schnell, A., Ashenberg, O., Su, C.W., Smillie, C., Shekhar, K., et al. (2018). T helper cell cytokines modulate intestinal stem cell renewal and differentiation. *Cell* 175, 1307–1320.e22. <https://doi.org/10.1016/j.cell.2018.10.008>.
- Linares, J.F., Zhang, X., Martinez-Ordoñez, A., Duran, A., Kinoshita, H., Kasashima, H., Nakanishi, N., Nakanishi, Y., Carelli, R., Cappelli, L., et al. (2021). PKClambda/iota inhibition activates an ULK2-mediated interferon response to repress tumorigenesis. *Mol. Cell* 81, 4509–4526.e10. <https://doi.org/10.1016/j.molcel.2021.08.039>.
- Llado, V., Nakanishi, Y., Duran, A., Reina-Campos, M., Shelton, P.M., Linares, J.F., Yajima, T., Campos, A., Aza-Blanc, P., Leitges, M., et al. (2015). Repression of intestinal stem cell function and tumorigenesis through direct phosphorylation of beta-catenin and yap by PKCzeta. *Cell Rep.* 10, 740–754. <https://doi.org/10.1016/j.celrep.2015.01.007>.
- Tetteh, P.W., Basak, O., Farin, H.F., Wiebrands, K., Kretschmar, K., Begthel, H., van den Born, M., Korving, J., de Sauvage, F., van Es, J.H., et al. (2016). Replacement of Lost Lgr5-Positive Stem Cells through Plasticity of Their Enterocyte-Lineage Daughters. *Cell Stem Cell* 18, 203–213. <https://doi.org/10.1016/j.stem.2016.01.001>.
- Nakanishi, Y., Reina-Campos, M., Nakanishi, N., Llado, V., Elmen, L., Peterson, S., Campos, A., De, S.K., Leitges, M., Ikeuchi, H., et al. (2016). Control of Paneth Cell Fate, Intestinal Inflammation, and Tumorigenesis by PKClambda/iota. *Cell Rep.* 16, 3297–3310. <https://doi.org/10.1016/j.celrep.2016.08.054>.
- Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369, 156–160. <https://doi.org/10.1038/369156a0>.
- Dérjard, B., Hibi, M., Wu, I.H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 76, 1025–1037. [https://doi.org/10.1016/0092-8674\(94\)90380-8](https://doi.org/10.1016/0092-8674(94)90380-8).
- Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Dérjard, B., Davis, R.J., Johnson, G.L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* 266, 1719–1723. <https://doi.org/10.1126/science.7992057>.
- Kudo, Y., Sugimoto, M., Arias, E., Kasashima, H., Cordes, T., Linares, J.F., Duran, A., Nakanishi, Y., Nakanishi, N., L'Hermitte, A., et al. (2020). PKClambda/iota loss induces autophagy, oxidative phosphorylation, and NRF2 to promote liver cancer progression. *Cancer Cell* 38, 247–262.e11. <https://doi.org/10.1016/j.ccell.2020.05.018>.
- Vasquez, E.G., Nasreddin, N., Valbuena, G.N., Mulholland, E.J., Belnoue-Davis, H.L., Egginton, H.R., Schenck, R.O., Wouters, V.M., Wirapati, P., Gilroy, K., et al. (2022). Dynamic and adaptive cancer stem cell population admixture in colorectal neoplasia. *Cell Stem Cell* 29, 1213–1228.e8. <https://doi.org/10.1016/j.stem.2022.07.008>.

28. Du, J., Sarkar, R., Li, Y., He, L., Kang, W., Liao, W., Liu, W., Nguyen, T., Zhang, L., Deng, Z., et al. (2022). N(6)-adenomethylation of GsdmC is essential for Lgr5(+) stem cell survival to maintain normal colonic epithelial morphogenesis. *Dev. Cell* 57, 1976–1994.e8. <https://doi.org/10.1016/j.devcel.2022.07.006>.
29. Mustata, R.C., Vasile, G., Fernandez-Vallone, V., Strollo, S., Lefort, A., Libert, F., Monteyne, D., Pérez-Morga, D., Vassart, G., and Garcia, M.I. (2013). Identification of Lgr5-independent spheroid-generating progenitors of the mouse fetal intestinal epithelium. *Cell Rep.* 5, 421–432. <https://doi.org/10.1016/j.celrep.2013.09.005>.
30. Leach, J.D.G., Vlahov, N., Tsantoulis, P., Ridgway, R.A., Flanagan, D.J., Gilroy, K., Sphyrin, N., Vázquez, E.G., Vincent, D.F., Faller, W.J., et al. (2021). Oncogenic BRAF, unrestrained by TGFbeta-receptor signalling, drives right-sided colonic tumorigenesis. *Nat. Commun.* 12, 3464. <https://doi.org/10.1038/s41467-021-23717-5>.
31. Bala, P., Rennhack, J.P., Aitymbayev, D., Morris, C., Moyer, S.M., Duronio, G.N., Doan, P., Li, Z., Liang, X., Hornick, J.L., et al. (2023). Aberrant cell state plasticity mediated by developmental reprogramming precedes colorectal cancer initiation. *Sci. Adv.* 9, eadf0927. <https://doi.org/10.1126/sciadv.adf0927>.
32. Giroux, V., and Rustgi, A.K. (2017). Metaplasia: tissue injury adaptation and a precursor to the dysplasia-cancer sequence. *Nat. Rev. Cancer* 17, 594–604. <https://doi.org/10.1038/nrc.2017.68>.
33. Lee, H.O., Hong, Y., Etioglu, H.E., Cho, Y.B., Pomella, V., Van den Bosch, B., Vanhecke, J., Verbandt, S., Hong, H., Min, J.W., et al. (2020). Lineage-dependent gene expression programs influence the immune landscape of colorectal cancer. *Nat. Genet.* 52, 594–603. <https://doi.org/10.1038/s41588-020-0636-z>.
34. Uhlitz, F., Bischoff, P., Peidli, S., Sieber, A., Trinks, A., Lüthen, M., Obermayer, B., Blanc, E., Ruchiy, Y., Sell, T., et al. (2021). Mitogen-activated protein kinase activity drives cell trajectories in colorectal cancer. *EMBO Mol. Med.* 13, e14123. <https://doi.org/10.15252/emmm.202114123>.
35. Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA* 102, 15545–15550. <https://doi.org/10.1073/pnas.0506580102>.
36. Mootha, V.K., Lindgren, C.M., Eriksson, K.F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstråle, M., Laurila, E., et al. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat. Genet.* 34, 267–273. <https://doi.org/10.1038/ng1180>.
37. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. <https://doi.org/10.1093/bioinformatics/bts635>.
38. Zhang, Y., Liu, T., Meyer, C.A., Eeckhoutte, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., and Liu, X.S. (2008). Model-based analysis of ChIP-Seq (MACS). *Genome Biol* 9, R137. <https://doi.org/10.1186/gb-2008-9-9-r137.3>.
39. Yu, G., Wang, L.G., and He, Q.Y. (2015). ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. *Bioinformatics* 31, 2382–2383. <https://doi.org/10.1093/bioinformatics/btv145.4>.
40. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. <https://doi.org/10.1038/nmeth.1923>.
41. Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., 3rd, Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data. *Cell* 177, 1888–1902.e1821. <https://doi.org/10.1016/j.cell.2019.05.031>.
42. Gulati, G.S., Sikandar, S.S., Wesche, D.J., Manjunath, A., Bharadwaj, A., Berger, M.J., Ilagan, F., Kuo, A.H., Hsieh, R.W., Cai, S., et al. (2020). Single-cell transcriptional diversity is a hallmark of developmental potential. *Science* 367, 405–411. <https://doi.org/10.1126/science.aax0249>.
43. Korsunsky, I., Millard, N., Fan, J., Slowikowski, K., Zhang, F., Wei, K., Baglaenko, Y., Brenner, M., Loh, P.R., and Raychaudhuri, S. (2019). Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat. Methods* 16, 1289–1296. <https://doi.org/10.1038/s41592-019-0619-0>.
44. Madison, B.B., Dunbar, L., Qiao, X.T., Braunstein, K., Braunstein, E., and Gumucio, D.L. (2002). Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J. Biol. Chem.* 277, 33275–33283. <https://doi.org/10.1074/jbc.M204935200>.
45. el Marjou, F., Janssen, K.P., Chang, B.H.J., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robine, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* 39, 186–193. <https://doi.org/10.1002/gene.20042>.
46. George, J., Mittal, S., Kadamberi, I.P., Pradeep, S., and Chaluvally-Raghavan, P. (2022). Optimized proximity ligation assay (PLA) for detection of RNA-protein complex interactions in cell lines. *Star Protoc.* 3, 101340. <https://doi.org/10.1016/j.xpro.2022.101340>.
47. Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* 36, 411–420. <https://doi.org/10.1038/nbt.4096>.
48. Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., and Glass, C.K. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell* 38, 576–589. <https://doi.org/10.1016/j.molcel.2010.05.004>.
49. Li, Z., Schulz, M.H., Look, T., Begemann, M., Zenke, M., and Costa, I.G. (2019). Identification of transcription factor binding sites using ATAC-seq. *Genome Biol.* 20, 45. <https://doi.org/10.1186/s13059-019-1642-2>.
50. Zhang, K., Wang, M., Zhao, Y., and Wang, W. (2019). Taiji: system-level identification of key transcription factors reveals transcriptional waves in mouse embryonic development. *Sci. Adv.* 5, eaav3262. <https://doi.org/10.1126/sciadv.aav3262>.
51. Bankhead, P., Loughrey, M.B., Fernández, J.A., Dombrowski, Y., McArt, D.G., Dunne, P.D., McQuaid, S., Gray, R.T., Murray, L.J., Coleman, H.G., et al. (2017). QuPath: open source software for digital pathology image analysis. *Sci. Rep.* 7, 16878. <https://doi.org/10.1038/s41598-017-17204-5>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti- β -actin	Sigma-Aldrich	Cat# A1978, RRID: AB_476692
Rabbit anti-aPKCs	Abcam	Cat# ab59364; RRID: AB_944858
Mouse anti-PKC λ	BD Biosciences	Cat# 610208; RRID: AB_397607
Rabbit anti-PKC ζ	Cell Signaling Technology	Cat# 9368; RRID: AB_10693777
Rabbit anti-PKC ζ	ProteinTech	Cat# 26899-1-AP, RRID: AB_2880675
Rabbit anti-Cleaved Caspase-3	Cell Signaling Technology	Cat# 9664, RRID: AB_2070042
Rabbit anti-Ki67	Abcam	Cat# ab16667, RRID: AB_302459
Rabbit anti-OLFM4	Cell Signaling Technology	Cat# 39141, RRID: AB_2650511
Rabbit anti-MUC5AC	Cell Signaling Technology	Cat# 61193, RRID: AB_2799603
Rabbit anti-ANXA10	Abcam	Cat# ab213656, RRID: AB_2921231
Mouse anti-ANXA10	Santa Cruz Biotechnology	Cat# sc-398736, RRID: AB_3076229
Rabbit anti-CLU	Abcam	Cat# ab184100, RRID: AB_2892532
Goat anti-CLU	R&D systems	Cat# AF2747, RRID: AB_2083314
Rabbit anti-YAP	Cell Signaling Technology	Cat# 14074, RRID: AB_2650491
Rabbit anti-phospho-cJun	Cell Signaling Technology	Cat# 2361, RRID: AB_490908
Rabbit anti-phospho-cJun	Cell Signaling Technology	Cat# 3270, RRID: AB_2129575
Rabbit anti-phospho-JNK	Cell Signaling Technology	Cat# 4668, RRID: AB_823588
Rabbit anti-GFP	Cell Signaling Technology	Cat# 2956, RRID: AB_1196615
Chicken anti-GFP	Abcam	Cat# ab13970, RRID: AB_300798
Rabbit anti-c-JUN	Cell Signaling Technology	Cat# 9165, RRID: AB_2130165
Rabbit anti-c-JUN	Santa Cruz Biotechnology	Cat# sc-1694, RRID: AB_631263
Mouse anti-TEAD	BD Biosciences	Cat# 610922, RRID: AB_398237
Rabbit anti-Lysozyme	DAKO	Cat# A0099, RRID: AB_2341230
Goat anti-Mouse IgG1, secondary, HRP	Thermo Fisher Scientific	Cat# PA1-74421, RRID: AB_10988195
Goat anti-Rabbit IgG, secondary, HRP	Thermo Fisher Scientific	Cat# 31461, RRID: AB_228347
Mouse anti-Goat IgG, secondary, HRP	Santa Cruz Biotechnology	Cat# sc-2354, RRID: AB_628490
Goat anti-Rabbit, secondary, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A11008, RRID: AB_143165
Goat anti-Mouse IgG, secondary, Alexa Fluor 568	Thermo Fisher Scientific	Cat# A11004, RRID: AB_2434072
Goat anti-Mouse IgG1, secondary, Alexa Fluor 568	Thermo Fisher Scientific	Cat# A21124, RRID: AB_2535766
Donkey anti-Mouse IgG, secondary, Alexa Fluor 647	Thermo Fisher Scientific	Cat# A31571, RRID: AB_162542
Goat anti-Rabbit IgG, secondary, IRDye 800	LI-COR Biosciences	Cat# 926-32211, RRID: AB_621843
Goat anti-Mouse IgG1, secondary, IRDye 800	LI-COR Biosciences	Cat# 926-32350, RRID: AB_2782997
Goat anti-Mouse IgG, secondary, IRDye 800	LI-COR Biosciences	Cat# 926-32210, RRID: AB_621842
Biological samples		
Human Colorectal cancers (CRCs)	Osaka City University Hospital, Osaka, JAPAN	N/A
Human Colorectal cancers (CRCs)	Santa Lucía General University Hospital (HGUSL), Cartagena, SPAIN	N/A
Human colon samples (tubular adenomas, sessile serrated lesions, traditional serrated adenomas)	Santa Lucía General University Hospital (HGUSL), Cartagena, SPAIN	N/A
Chemicals, peptides, and recombinant proteins		
Ampicillin	Fisher BioReagents	Cat# BP1760-25
Neomycin	Sigma-Aldrich	Cat# N6386

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Vancomycin	Sigma-Aldrich	Cat# V2002
Puromycin	Omega Scientific, inc.	Cat# PR-01
X-tremeGENE HP DNA Transfection Reagent	Roche	Cat# 6366236001
Metronidazole	Sigma-Aldrich	Cat# M3761
HBSS (no calcium, no magnesium)	GIBCO	Cat# 14175095
Dimethyl Sulfoxide	Fisher BioReagents	Cat# BP2311
Chloroform	Sigma-Aldrich	Cat# 288306
Methanol	Sigma-Aldrich	Cat# 1424109
Tryple Express	Life Technologies	Cat# 12605-010
HEPES	Gibco	Cat# 15630080
PBS (no calcium, no magnesium)	Gibco	Cat# 10010-023
Quick-RNA Miniprep Kit	Zymo Research	Cat# R1054
RNAlater Stabilization Solution	Invitrogen	Cat# AM7021
TRIzol	Thermo Fisher Scientific	Cat# 15596018
Advanced DMEM/F12	Thermo Fisher Scientific	Cat# 12634010
Opti-MEM Reduced Serum Medium	Gibco	Cat# 31985070
DMEM	Corning	Cat# 10-017CV
GlutaMAX Supplement	Thermo Fisher Scientific	Cat# 35050061
Penicillin-Streptomycin solution	Corning	Cat# 30-002-CI
Y-27632	Tocris	Cat# 1254
B27 Supplement	Thermo Fisher Scientific	Cat# 17504001
N2 Supplement	Thermo Fisher Scientific	Cat# 17502048
Human EGF	Peptotech	Cat#AF-100-15
Recombinant murine Noggin	Peptotech	Cat# 250-38
Human IGF-1	Biologend	Cat#590906
Human FGF-2	Peptotech	Cat#100-18B
A83-01	Sigma-Aldrich	Cat#SML078
Human [Leu15]-Gastrin I	Sigma-Aldrich	Cat# G9145
Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix	Corning	Cat# 356230
Dispase II	Sigma-Aldrich	Cat# #D4693
DNase I	Sigma-Aldrich	Cat# 04716728001
4-OH-tamoxifen (4-OHT)	Millipore-Sigma	Cat# H7904
TrueCut Cas9 Protein v2	Thermo Fisher Scientific	Cat# A36498
DAPI	Life Technologies	Cat# D1306
Alcian blue	Millipore-Sigma	Cat# B8438
Nuclear Fast red solution	Millipore-Sigma	Cat# N3020
Blue Alkaline Phosphatase	Vector laboratorial	Cat# SK-5300
Trypan Blue Solution, 0.4%	Gibco	Cat# 15250061
JNK-IN-8	Selleckchem	Cat# S4901
QVD-OPH	MedKoo Biosciences	Cat# 526845
Verteporfin	Sigma-Aldrich	Cat# SML0534
Z-VAD-FMK	Selleckchem	Cat# S7023
GSK'872	Selleckchem	Cat# S8465
N-Acetyl Cystein (NAC)	Sigma-Aldrich	Cat# A7250
Propidium iodide	Sigma-Aldrich	Cat# P4170
Butylated hydroxyanisole (BHA)	Sigma-Aldrich	Cat# B1253
Critical commercial assays		
Neon™ 10µl Electroporation Kit	Thermo Fisher Scientific	Cat# MPK1096

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chromium Single Cell 3' Gene Expression Solution v2	10X Genomics	Cat#PN-120237
EasySep™ Dead Cell Removal (Annexin V) Kit	STEMCELL Technologies	Cat# 17899
VECTASTAIN® Elite® ABC-HRP Kit	Vector	Cat# PK-6100
Opal 3-Plex Manual Detection Kit	AKOYA BIOSCIENCES	Cat# NEL810001KT
In situ Cell Death Detection Kit, TMR red	Roche	Cat# 12156792910
RNAscope® 2.5 HD Detection Reagents-BROWN	ACD	Cat# 322310
CellTiter-Glo® 3D Cell Viability Assay	Promega	Cat# G9681
Duolink In situ PLA kit: Duolink In Situ Detection Reagents Red	Sigma-Aldrich	Cat #Duo92008
Duolink In situ PLA kit: Duolink In Situ PLA Probe Anti-Rabbit PLUS	Sigma-Aldrich	Cat #Duo92002
Duolink In situ PLA kit: Duolink In Situ PLA Probe Anti-Mouse MINUS	Sigma-Aldrich	Cat #Duo92004

Deposited data

RNA-seq (mouse intestinal epithelial cells and tissues)	Nakanishi et al. ¹³	GEO: GSE109289
sc-RNA-seq	Martinez-Ordoñez et al. ¹⁷	GEO: GSE207780
Single cell 3' RNA sequencing from patients with CRC	Lee et al. ³³	GEO: GSE132465
Single cell 3' RNA sequencing from patients with CRC	Uhlitz et al. ³⁴	GEO: GSE166555
TCGA-COREAD	cBioportal	http://www.cbioportal.org/index.do
sc-RNA-seq	This study	GEO: GSE236848
RNA-seq	This study	GEO: GSE236649
ATAC-seq	Linares et al. ¹⁹	GEO: GSE180223
ATAC-seq	This study	GEO: GSE236850
Raw Data	This study; Mendeley Data	https://doi.org/10.17632/g67yy3t252.1

Experimental models: Organisms/strains

Mouse: <i>Prkcz</i> ^{fl/fl} ; <i>Villin-Cre</i>	Llado et al. ²⁰	N/A
Mouse: <i>Prkci</i> ^{fl/fl} ; <i>Villin-Cre</i>	Nakanishi et al. ²²	N/A
Mouse: <i>Prkci</i> ^{fl/fl} ; <i>Prkcz</i> ^{fl/fl} ; <i>Villin-Cre</i>	Nakanishi et al. ¹³	N/A
Mouse: <i>Apc</i> ^{fl/+} ; <i>Villin-Cre</i>	Nakanishi et al. ²²	N/A
Mouse: <i>Prkci</i> ^{fl/fl} ; <i>Apc</i> ^{fl/+} ; <i>Villin-Cre</i>	Nakanishi et al. ²²	N/A
Mouse: <i>Lgr5-EGFP-IRES-CreERT2</i>	The Jackson Laboratory	Stock No: 008875
Mouse: <i>Villin-Cre</i>	The Jackson Laboratory	Stock No: 004586
Mouse: <i>Villin-CreERT2</i>	The Jackson Laboratory	Stock No: 020282

Experimental models: Cell lines

Mouse MODE-K (sg <i>Prkci</i>)	Nakanishi et al. ²²	N/A
HEK293T	ATCC	Cat# CRL-3216, RRID:CVCL_0063
Human CRC (sg <i>PRKCI</i> / <i>PRKCZ</i>)	Martinez-Ordoñez et al. ¹⁷	N/A

Oligonucleotides

Real-time PCR primers	N/A	Table S1
RNAscope® Probe- Mm- <i>Lgr5</i>	ACD	Cat# 312171
RNAscope® Positive Control Probe- Mm- <i>Ppib</i>	ACD	Cat# 313911
RNAscope® Negative Control Probe- <i>DapB</i>	ACD	Cat# 310043

Recombinant DNA

pLKO.1 puro NT (non target)	Sigma-Aldrich	Cat# SHC016
pLKO.1 puro YAP1	Sigma-Aldrich	Cat# TRCN0000107266 (NM_006106)
Lentiviral packaging plasmid pMD2.G	Addgene	Cat# 12259

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Lentiviral packaging plasmid psPAX2	Addgene	Cat# 12260
Software and algorithms		
Graphpad Prism 8	Graphpad	https://www.graphpad.com/
QuPath v.0.1.3	Queen's University Belfast, Northern Ireland	https://qupath.github.io
ImageJ	NIH	https://imagej.nih.gov/ij/
BioRender	BioRender	https://biorender.com/
Zen blue	Zeiss	https://www.micro-shop.zeiss.com/en/us/softwarefinder/software-categories/zen-blue/
RStudio (1.1.456)	R Core Team	https://www.r-project.org/
R	R Core Team	https://www.r-project.org/
GSVA; version 1.26.0	Bioconductor	https://github.com/rcastelo/GSVA
GSEA (v4.1.0)	Broad Institute	http://www.broadinstitute.org/gsea/index.jsp
GSEA Webapp	Subramanian et al. ³⁵ and Mootha et al. ³⁶	https://www.gsea-msigdb.org/gsea/index.jsp
GenePattern	Broad Institute	https://cloud.genepattern.org/gp/pages/login.jsf
IGV(v2.4.13)	Broad Institute	https://igv.org/
STAR	Dobin et al. ³⁷	https://github.com/alexdobin/STAR
MACS2 (2.1.0)	Zhang et al. ³⁸	https://github.com/taoliu/MACS
ChIPSeeker (3.6)	Yu et al. ³⁹	https://github.com/GuangchuanYu/ChIPseeker
Cell Ranger (v3.0)	Langmead et al. ⁴⁰ ; 10X genomics	http://software.10xgenomics.com/single-cell/overview/welcome
Seurat 3.0	Stuart et al. ⁴¹	https://github.com/Satijalab/seurat
CytoTRACE	Gulati et al. ⁴²	https://cytotrace.stanford.edu
Harmony	Korsunsky et al. ⁴³	https://portals.broadinstitute.org/harmony/articles/quickstart.html
PhenoChart Whole Slide Viewer	AKOYA BIOSCIENCES	https://www.akoyabio.com/support/software/phenochart-whole-slide-viewer/
CaseViewer	3DHistech, Budapest, Hungary	https://www.3dhistech.com/solutions/caseviewer/
Other		
EVOS FL Auto Imaging System	Thermo Fisher Scientific	N/A
EVOS M5000 Imaging System	Thermo Fisher Scientific	N/A
TissueLyser II	QIAGEN	Cat# 85300
NanoDrop 1000 spectrophotometer	Thermo Fisher Scientific	N/A
Zeiss LSM 710 NLO Confocal Microscope	Zeiss	N/A
Neon™ Transfection System	Thermo Fisher Scientific	Cat# MPK5000
Panoramic Scanner	3DHistech, Budapest, Hungary	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jorge Moscat (jom4010@med.cornell.edu).

Materials availability

Cell and mouse lines generated in this study are available from the [lead contact](#) upon request with a completed Materials Transfer Agreement.

Data and code availability

The RNA-seq, scRNA-seq, and ATAC-seq datasets generated during this study have been deposited to the GEO repository on the NCBI website (GEO: GSE236851). This paper reports data derived from published and publicly available datasets GEO: GSE109289, GSE207780, GSE132465, GSE180223, and GSE166555. These datasets are located on the [key resources table](#). Original raw data have been deposited in Mendeley Data: <https://doi.org/10.17632/g67yy3t252.1> Any additional information required to reanalyze the data reported in this work paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

Animal handling and experimental procedures conformed to institutional guidelines and were approved by the Sanford-Burnham-Prebys Medical Discovery Institute Institutional Animal Care and Use Committee, and by the Weill Cornell Medicine Institutional Animal Care and Use Committee. *Prkcz*^{fl/fl}; *Villin-Cre*, *Prkci*^{fl/fl}; *Villin-Cre*, *Prkci*^{fl/fl}; *Prkcz*^{fl/fl}; *Villin-Cre*, *Prkci*^{fl/fl}; *Villin-CreERT2*, *Prkci*^{fl/fl}; *Prkcz*^{fl/fl}; *Villin-CreERT2*, *Prkci*^{fl/fl}; *Lgr5-EGFP-IRES-CreERT2*, *Apc*^{fl/+}; *Villin-Cre* and *Prkci*^{fl/fl}; *Apc*^{fl/+}; *Villin-Cre* mice were previously described.^{13,17,19,20,22,44,45} All mouse strains were generated in a C57BL/6 background and were born and maintained under pathogen-free conditions. For antibiotic treatment, 10-week-old *Prkci*^{fl/fl}; *Prkcz*^{fl/fl}; *Villin-Cre* mice were administered a combination of ampicillin (1 g/L), neomycin (1 g/L), metronidazole (1 g/L), and vancomycin (0.5 g/L) in drinking water for 4 weeks, until they were sacrificed. To induce *CreERT2*-mediated recombination, 8- to 12-week-old mice received one dose of 6 mg or three daily doses of 2 mg of TAM in corn oil by intraperitoneal injection. For TAM induction of the *Prkci*^{fl/fl}; *Lgr5-EGFP-IRES-CreERT2* model, 8- to 12-week-old mice received one dose of 6 mg for days 1, 5, and 11, and twelve doses of 2 mg every other day for the time-point of day 24. For the intestinal regeneration experiments, mice were exposed to whole-body irradiation (10 Gy) from a Caesium 137 irradiator and were analyzed 1 week later. All the mice were maintained on food and water ad libitum and were age-matched and co-housed for all experiments. All genotyping was done by PCR. Age- and sex-matched mice were used for all experiments.

Human samples

For CRC samples, a total of 490 CRC patients who had undergone a resection of the primary tumor at Osaka City University Hospital and Santa Lucía General University Hospital (HGUSL) of Cartagena, Spain were analyzed. CRC tissues were obtained from each patient, and none had undergone preoperative radiation or chemotherapy. For colonic adenoma samples, a total of 54 samples from 35 patients who had undergone polyp resection were obtained from Santa Lucía General University Hospital (HGUSL). This study was approved by the Osaka City University Ethics Committee and the HSUGL ethical board and written informed consent was obtained from patients. De-identified human samples were sent to Weill Cornell Medicine and used for histological analyses. The study was approved by the IRB Committee of Weill Cornell Medicine.

METHOD DETAILS

Intestinal epithelial cell isolation and intestinal organoid culture

IEC isolation and intestinal organoid culture were performed as described previously with some modifications.²⁰ Briefly, small intestines were removed, washed with cold PBS, opened longitudinally, cut into 5 mm fragments, and then washed several times with cold PBS until clean. Cut tissue fragments were incubated with 5 mM EDTA in PBS containing 10% FBS for 40 min at 4°C. IECs were then mechanically separated from the connective tissue by rigorous shaking, and then filtered through a 100-μm mesh into a 50 mL conical tube to remove tissue fragments. Isolated IECs were pelleted and then lysed for RNA or protein extraction. For intestinal organoid culture, cut tissue fragments were incubated with 8 mM EDTA in PBS for 15 min at room temperature, shaken briefly to remove villi, incubated with fresh 8 mM EDTA in PBS for another 15 min at 4°C and shaken vigorously to isolate crypts. Around 300 crypts were mixed with 50 μL of Matrigel and plated in 24-well plates. After polymerization of Matrigel, 0.5 mL of ENR or WENR medium (Advanced DMEM-F12 containing 10 mM HEPES, 1X Glutamax, 1X B27 supplement, 50 ng/mL EGF, 100 ng/mL Noggin, 10% R-spondin-1 conditioned medium and 10 mM Y-27632 with or without 50% WNT3A conditioned medium) was added. Organoids were maintained by refreshing medium every other day and passaged as needed. For the induction of Cre-mediated recombination, 500 nM 4-OH tamoxifen was added to the media for the duration of each experiment. In the indicated experiments, JNK-IN-8 (0.5 or 1 μM), Q-VD-Oph (30 μM), Verteporfin (2.5 or 5 μM), or zVAD-fmk (10 μM) with GSK'872 (2 μM) were added to the medium at the same time with 4-OH tamoxifen. For the propidium-iodide (PI)-traced cell-death assay, 1 μg/mL PI was added in the medium 30 min before taking images. Patient-derived organoids (PDOs) were established as previously described¹⁷ and cultured in advanced DMEM/F12 supplemented with, 50 ng/mL recombinant human EGF, 100 ng/mL recombinant murine Noggin, 500 nM A83-01, 100 ng/mL recombinant human IGF-1, 50 ng/mL recombinant human FGF-2, 10 μM Y-27632, 1X B27 supplement, 10 nM human [Leu15]-Gastrin I, 1X GlutaMAX, 1X, and 1X penicillin/streptomycin (ENAI medium). Knockdown of YAP1 in PDOs, was achieved by lentivirus-mediated transduction. TRC lentiviral shRNA targeting human YAP1 (TRCN0000107266) was obtained from Sigma-Aldrich. shRNA-encoding plasmids were co-transfected with psPAX2 (Addgene) and pMD2.G (Addgene) plasmids into HEK293T cells by using XtremeGene HP transfection reagent (Roche). Virus-containing supernatants were collected 48 h after transfection, filtered to eliminate cells, and supplemented with 8 μg/mL polybrene. Organoids were infected by spinoculation with viral supernatants and selected with

puromycin (3 μ g/mL). Treatment of MODE-K cells sgC or sgPrkci with the ROS scavengers, N-acetylcysteine (NAC) or butylated hydroxyanisole (BHA) from sigma at 5 mM and 100 μ M respectively for 48 h.

Histology, Immunohistochemistry, Immunofluorescence, and in situ hybridization (ISH)

Tissues from indicated mice were isolated, rinsed in ice-cold PBS, fixed in 10% neutral buffered formalin overnight at 4°C, dehydrated, and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin. For immunohistochemistry, sections were deparaffinized, rehydrated, and then treated for antigen retrieval. After blocking in Protein Block Serum-Free solutions (DAKO), tissues were incubated with primary antibody overnight at 4°C followed by incubation with biotinylated secondary antibody. Endogenous peroxidase was quenched in 3% H₂O₂ in water for 10 min at room temperature. Antibodies were visualized with avidin-biotin complex (Vectastain Elite; Vector Laboratories) using diaminobenzidine as the chromogen. Cell death was analyzed using the in situ cell death detection kit, TMR red (Roche) for TUNEL staining. Alkaline phosphatase (ALP) staining was performed using a kit from Vector Laboratories (SK-5300) following the manufacturer's instructions. Briefly, slides were deparaffinized and incubated with ALP detection solution for 30 minutes. Nuclei were stained using a Nuclear Fast Red Solution (Sigma, N3020). Goblet cells were detected using an Alcian blue solution (Sigma, B8438). For immunofluorescence, the signal was visualized using the OPAL™ 3-Plex Manual IHC Kit (Akoya Biosciences, MA), which allows simultaneous detection of multiple targets in the same image. Fluorophores Opal 520, Opal 570, and TSA Plus Cyanine 5 were used, and the sections were counterstained with Spectral DAPI. ISH was performed by using RNAscope 2.5 HD Reagent Kit Brown, following the manufacturer's protocols. Stained sections were analyzed with a Zeiss light microscope or a Zeiss 880 laser scanning confocal microscope supplemented with Zen 3.3 Blue edition software.

In situ proximity ligation assay

In situ PLA was performed with DuoLink In Situ Reagents Sigma. MODE-K sgC and sgPrkci cells were seeded in fibronectin-coated glass chamber slides. 48 h later, the cells were fixed in 4% PFA for 10 min at room temperature. Proximity ligation assays were performed as indicated by the provider's protocol, after an overnight incubation with primary antibodies following the immunofluorescence protocol for cell cultures described by Abcam. Images were acquired with a Zeiss LSM 710 NLO Confocal microscope. Images were processed using Image J software (NIH) and the PLA signal was quantified as described.⁴⁶ Primary antibodies are listed in the [key resources table](#).

Immunoblotting Analysis

Cells for protein analysis were lysed in RIPA buffer (20 mM Tris-HCl, 37 mM NaCl, 2 mM EDTA, 1% Triton-X, 10% glycerol, 0.1% SDS, and 0.5% sodium deoxycholate) with phosphatase and protease inhibitors. Protein concentration in lysates was determined using Protein Assay Kit (Bio-Rad). Cell extracts were denatured, subjected to SDS-PAGE, transferred to PVDF membranes (GE Healthcare). After blocking with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween (TBS-T), the membranes were incubated with the specific antibodies (as listed in [key resources table](#)) overnight at 4°C. After 2 h incubation with the appropriate fluorochrome-conjugated antibodies, the immune complexes were detected by Near-infrared fluorescence (LI-COR).

RNA Extraction and Analysis

Total RNA from cultured organoids was extracted using TRIZOL reagent (Invitrogen), and purified by the RNeasy Mini Kit (QIAGEN), followed by DNase treatment. After quantification using a Nanodrop 1000 spectrophotometer (Thermo Scientific), RNA was either processed for RNA-seq or reverse-transcribed using random primers and MultiScribe Reverse Transcriptase (Applied Biosystems). Gene expression was analyzed by amplifying 500 ng of the complementary DNA using the CFX96 Real Time PCR Detection System with SYBR Green Master Mix (BioRad) and primers described in [Table S1](#). The amplification parameters were set at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s (40 cycles total). Gene expression values for each sample were normalized to the 18S RNA.

Chromatin Immunoprecipitation (ChIP)

MODE-K cells were fixed by adding directly to the culture medium formaldehyde (HCHO; from a 37% HCHO–10% methanol stock, Calbiochem) to a final concentration of 1%. After, 20 min of incubation with 125 mM glycine, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS, and protease inhibitors and incubated for 30 min at 4°C. Chromatin was sheared in a COVARIS S220 Focused-ultrasonicator to yield DNA fragment sizes of 200–1000 base pairs, and diluted 10 times in dilution buffer (20 mM Tris pH 8.0, 2 mM EDTA, 1% triton X-100, 150 mM NaCl and protease inhibitors). Immunoprecipitations were carried out overnight at 4°C using the following protein A-antibodies complexes: TEAD1 (Cat# 610922, RRID:AB_398237) and c-JUN (Cat# 9165, RRID: AB_2130165). Immunocomplexes were washed three times with buffer TSEI (20 mM Tris pH 8.0, 2 mM EDTA, 1% triton X-100, 150 mM NaCl, 0.1% SDS and protease inhibitors), three washes with buffer TSEII (20 mM Tris pH 8.0, 2 mM EDTA, 1% triton X-100, 500 mM NaCl, 0.1% SDS and protease inhibitors), one wash with buffer TSEIII (10 mM Tris pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% NP40, 1% Deoxycholate and protease inhibitors) and one wash with TE pH 8.0. Immunocomplexes were extracted in TE containing 1% SDS, and protein–DNA cross-links were reverted by heating at 65°C overnight. DNA was extracted by using a PCR purification kit (Qiagen). One-tenth of the immunoprecipitated DNA was used in each PCR, for which the promoter-specific primers ([Table S1](#)) were used.

ATAC-seq library preparation and sequencing

The cell pellet was resuspended in 50 μ L lysis buffer and then spun down 500 $\times$ g for 10 min at 4°C. The nuclei pellet was resuspended into 50 μ L transposition reaction mixture containing Tn5 transposase from Nextera DNA Library Prep Kit (Illumina) and incubated at 37°C for 30 min. Then the transposase-associated DNA was purified using MinElute PCR purification kit (QIAGEN). To amplify the library, the DNA was first amplified for 5 cycles using indexing primer from Nextera kit and NEBNext High-Fidelity 2X PCR master mix. To reduce the PCR amplification bias, 5 μ L of amplified DNA after the first 5 cycles was used to do qPCR of 20 cycles to decide the number of cycles for the second round of PCR. Usually, the maximum cycle of the second round of PCR is 5 cycles. Then the total amplified DNA was size selected into fragments less than 800 bp using SPRI beads. Quantification of the ATAC-seq library was done with QuBit. The size of the pooled library was examined by TapeStation. Barcoded ATAC-seq libraries were pooled and paired-end sequenced (sX75) on the Illumina NextSeq 500 using the High-output V2.5 kit (Illumina Inc., San Diego, CA).

10x Library Preparation and Sequencing

To prepare crypt-enriched IEC suspension, isolated crypts were digested with digestion buffer (0.4 mg/mL Dispase and 0.1 mg/mL DNase in HBSS) for 20 min at 37°C with agitation, filtered through 70- and 40- μ m-mesh cell strainers, washed with HBSS and resuspended with Advanced DMEM-F12 containing 10 mM HEPES, 1X Glutamax, 1X B27 supplement, 50 ng/mL EGF and 10 mM Y-27632. Dead cells were removed by EasySep Dead Cell Removal (Annexin V) kit (STEMCELL technologies). scRNA-seq libraries were generated using the Chromium Single Cell 30 Reagent Kit v2 (10X Genomics). Cells were loaded onto the 10X Chromium Single Cell Platform (10x Genomics) at a concentration of 2,000 cells per μ L (Single Cell 3'library and Gel Bead Kit v.2) as described in the manufacturer's protocol (10x User Guide, Revision B). Generation of gel beads in emulsion (GEMs), barcoding, GEM-RT clean-up, complementary DNA amplification, and library construction were all performed as per the manufacturer's protocol. Individual sample quality was checked using a Bioanalyzer TapeStation (Agilent). Qubit was used for library quantification before pooling. The final library pool was sequenced on an Illumina NovaSeq6000 instrument using an S1 flow cell.

Analysis of scRNA sequencing data

For scRNA-seq, raw sequence reads were quality-checked using FastQC software. The Cell Ranger version 2.1.1 software suite from 10x Genomics (<https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest>) was used to process, align and summarize unique molecular identifier (UMI) counts against the mouse mm10 assembly reference genome analysis set, obtained from the University of California Santa Cruz (UCSC). Raw, unfiltered count matrices were imported into R for further processing. Raw UMI count matrices were filtered using the Seurat v 3.0 R package⁴⁷ to remove: barcodes with very low (less than 200, empty wells) and very high (more than 4000, probably doublets) total UMI counts; matrices for which a high percentage of UMIs originated from mitochondrial features (more than 12%); and matrices for which fewer than 250 genes were expressed. Subsequently, the data were normalized using the SCTransform function, regressing out the following variables: total number of UMIs per cell and percentage of mitochondrial UMIs. Following normalization, the principal components were computed. The top principal components were identified using the ElbowPlot function and used for the UMAP dimensionality reduction. For clustering, we used Seurat in combination with Harmony software⁴³ to correct the potential effects of technical differences between sequencing batches. The RunHarmony(), RunUMAP(), FindNeighbors(), and FindClusters() functions were run for clustering and the percentage of mitochondrial features was considered to be a source of unwanted variation and was regressed out using the Seurat package. Genes specifically expressed in each cluster were identified with the FindAllMarkers() function and the Wilcoxon test labeling the different populations using the genes differentially up-regulated in each population. *Epcam*-negative cells were not considered, and the cell groups were annotated based on the marker gene analysis and canonical markers from the literature (Table S2). The scoring for the indicated signatures was performed using the AddModuleScore function in Seurat with default parameters. The WhichCells() function was run to identify double-positive, single-positive, or double-negative cell expression for YAP and AP-1 signatures. Single-cell RNA-seq data from *Prkci*^{fl/fl}*Prkcz*^{fl/fl}; *Villin*-Cre tumors (GSE207780) and CRC patients (GSE132465 and GSE166555) were downloaded from GEO Datasets and processed as described above. The TcTA, TRSC, and TFMC cell identification in human CRC were performed as described in¹⁷. Gene sets used for signature scoring are listed in Table S2 and when necessary, mapped to human orthologues using Ensembl BioMart. The visualization of the indicated signature score by Violin Plot and the statistical analysis (t-test) was performed using the ggplot2 package in R software.

CytoTRACE Analysis

CytoTRACE analysis was performed to compare differentiation states among stem cells in our dataset (<https://cytotrace.stanford.edu/>).⁴² This algorithm analyzes the number of uniquely expressed genes per cell, the distribution of mRNA content and the number of RNA copies per gene, to calculate a score assessing the differentiation and developmental potential of each cell. CytoTRACE analysis was performed using default parameters for stem cells from each genotype, the score was loaded to Seurat and represented as a ridge plot.

Bulk RNA-seq and ATAC-seq analysis

For RNA-seq, total RNA was extracted using Quick-RNA MiniPrep kit (Zymo Research), and library preparations and sequencing were conducted at GENEWIZ, LLC. Sequenced reads were trimmed to remove low-quality bases at ends and were then mapped to the reference genome (GRCm39) using STAR.

GenePattern (<https://cloud.genepattern.org/gp/pages/login.jsf>) was used to collapse gene matrix files (CollapseDataset module) or to assess the statistical significance of differential gene expression (DESeq2). Genes were sorted by $\log_2$ FC > 0.5 and adj > 0.05. Volcano plot representation for differentially expressed genes was generated using VolcanoR (<https://huygens.science.uva.nl/VolcanoR/>). Gene set enrichment analysis (GSEA) was performed using GSEA 4.0 software (<http://www.broadinstitute.org/gsea/index.jsp>) with 1000 gene-set permutations using the gene-ranking metric t-test with the collections h.all.v6.1.symbols (H), c2.all.v6.1.symbols (C2), c5.all.v6.1.symbols (C5), or customized gene signatures (Table S2). For ATAC-seq, FASTQ files from ATAC-seq reads were aligned to UCSC mm10 with Bowtie2 (bowtie2 -very-sensitive -x mm10 -1 FILE_merged_R1.fastq -2 FILE_merged_R2.fastq -X 1000 -p 12 | samtools view -u - | samtools sort - > FILE.bam). Peak calling was performed with MACS2 using -qvalue 0.05 -nomodel -shift -100 -extsize 200. Peaks were annotated with ChipSeeker R package. For motif enrichment analysis in the differential peaks between LKO or DKO versus WT or ZKO IECs, p values were calculated using findMotifsGenome.pl with -size given, -len 6,8,10,12 and -mask program of HOMER v4.10.3.⁴⁸ For footprinting analysis, rgt-hint differential command was used to infer transcription factor activity and to plot the results.⁴⁹ PageRank analysis combining ATAC-seq and RNA-seq was performed with Taiji v1.1.0.⁵⁰ “JDP2 target genes”, “JUND target genes” and “JUNB target genes” signatures were derived from predicted target genes of JDP2, JUND, and JUNB respectively in LKO and DKO IECs ATAC-seq, and with a positive $\log_2$ FC versus WT IECs in the RNA-seq. Transcription Factor motif analysis of *Anxa10* promoter was performed using JASPAR (<http://jaspar.genereg.net/>) and bigwig files from ATAC-seq data were visualized with IGV (<https://igv.org/>). RNA-seq data of The Cancer Genome Atlas (TCGA) Colorectal Adenocarcinoma (Tumor Samples with mRNA data (RNA Seq V2 RSEM), 592 tumor samples from 592 patients) was downloaded through cBioportal (<http://www.cbioportal.org/index.do>). TCGA CRC patients were separated into aPKC high and low as previously described.¹⁷ YAP and AP-1 score for each patient was calculated by computing the average of each signature using the gene set variation analysis (GSVA) algorithm, with the standard settings.

Tissue microarray analysis

Image analysis was processed in QuPath v0.1.3 (Queen’s University, Belfast, Northern Ireland). Scanned TMA slides were dearranged and preprocessed as previously described.⁵¹ After dearranging, all cores were examined and those with either no tumor or dysplastic region represented or with artifacts (tissue broken, for example) were excluded. Cell detection was determined for each core, and the total number of positive cells using DAPI staining as a cell reference was assessed. H-score, calculated based on the extent and intensity of the staining in epithelial (PKC λ /i, PKC ζ) was used for patient scoring. CRC or patients with adenomas were categorized into high and low PKC λ /i and PKC ζ expression groups using the H-score as the cut-off selected by an experienced pathologist. For OLFM4, samples were considered positive when at least 1%-30% of epithelial cells were stained. For YAP and p-cJUN, samples were considered positive when at least 1%-30% of epithelial cells were stained in the nuclei, whereas no signal detected was considered negative. For aPKCs and MUC5AC, patients were categorized as previously described.¹⁷

QUANTIFICATION AND STATISTICAL ANALYSIS

All the statistical tests were justified for every figure. All samples represent biological replicates. Data are presented as the mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism 8 or R software environment (<http://www.r-project.org/>). Pairwise differences were measured using two-tailed independent student’s t-tests. If the data did not meet this test, a Mann-Whitney U-test was used. Statistical significance between groups of 3 or more was determined by a one-way or two-way ANOVA, followed by Tukey’s multiple comparison test. The chi-square test or Fisher’s exact test was used to determine the significance of differences between covariates. Logistic regression analysis was employed to estimate univariate and multivariate odds ratio and 95% confidence interval (CI). Values of $p < 0.05$ were considered significantly different.