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FULL LENGTH ARTICLE

Monogenic deficiency in murine intestinal Cdc42 leads to mucosal inflammation that induces crypt dysplasia

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KEYWORDS
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Irradiation

Abstract CDC42 controls intestinal epithelial (IEC) stem cell (IESC) division. How aberrant CDC42 initiates intestinal inflammation or neoplasia is unclear. We utilized models of inflammatory bowel diseases (IBD), colorectal cancer, aging, and IESC injury to determine the loss of intestinal *Cdc42* upon inflammation and neoplasia. Intestinal specimens were collected to determine the levels of CDC42 in IBD or colorectal cancer. *Cdc42* floxed mice were crossed with *Villin*-Cre, *Villin*-CreER^{T2} and/or *Lgr5*-eGFP-IRES-CreER^{T2}, or *Bmi1*-CreER^{T2} mice to generate *Cdc42* deficient mice. Irradiation, colitis, aging, and intestinal organoid were used to evaluate CDC42 upon mucosal inflammation, IESC/progenitor regenerative capacity, and IEC repair. Our studies revealed that increased CDC42 in colorectal cancer correlated with lower survival; in contrast, lower levels of CDC42 were found in the inflamed IBD colon. Colonic *Cdc42* depletion significantly reduced Lgr5^{+} IESCs, increased progenitors' hyperplasia, and induced mucosal inflammation, which led to crypt dysplasia. Colonic *Cdc42* depletion markedly enhanced irradiation- or chemical-induced colitis. Depletion or inhibition of *Cdc42* reduced colonic Lgr5^{+} IESC regeneration. In conclusion, depletion of *Cdc42* reduces the IESC regeneration and IEC repair, leading to prolonged mucosal inflammation. Constitutive monogenic loss of *Cdc42* induces mucosal inflammation, which could result in intestinal neoplasia in the context of aging.

Introduction

Environmental factors, such as microbiota alterations or infection, can trigger an immune response that leads to intestinal mucosal injuries, which culminate in genetic changes and increased replication to alter intestinal epithelial stem cells (IESC) and progenitor homeostasis.[1,2] These changes in IESC/progenitors can break down epithelial barriers, resulting in gastrointestinal (GI) diseases, such as inflammatory bowel diseases (IBD) and colorectal cancer (CRC).[3–5] In North America and Europe, five million individuals are currently affected by IBD including Crohn's disease (CD) and ulcerative colitis (UC).[6] Chronic IBD or age-associated intestinal inflammation can directly induce the onset of CRC.[7] Altered IESC and progenitors play a direct role in driving chronic and relapsing mucosal inflammation in IBD to convert to colon cancer.[4,5] Although the mechanisms of this conversion remain enigmatic, therapeutic restoration of IESC–progenitor homeostasis is believed to improve clinical outcomes in both CD and UC patients and increase the sensitivity of colon cancers to therapies.[8,9]

Cell division cycle 42 (CDC42) is a small GTPase of the Rho family that establishes epithelial polarity through the PAR3-PAR6-atypical protein kinase C (aPKC) complex at the apical membrane.[10] Absence of CDC42 results in loss of epithelial polarity, epithelial specification, and tissue or organ formation.[11] Consistently, depletion of *Cdc42* impairs intestinal epithelial cell (IEC) differentiation into the functional intestinal barrier, contributing to chronic GI inflammation.[12] In contrast, CDC42 overexpression is associated with the development of invasive phenotypes in colon cancer.[13] *Cdc42* variants were recently determined as a susceptibility locus for developing CRC.[14] These findings suggest that CDC42 plays a critical role in GI inflammation and tumorigenesis. However, the role of CDC42 in disease progression from chronic inflammation to colorectal cancer progression and its association with therapy have not so far been sufficiently addressed, nor was its prognostic role illuminated and targeting explored.

Intriguingly, CDC42 may be also essential for adult IESC homeostasis.[15] *Cdc42*-deficient IESCs lose the ability to clonally expand and differentiate, further leading to disrupted intestinal crypt–villus axis.[12,15] It is assumed that intestinal responses to gut injury, regeneration, and tumorigenesis are controlled by IESCs at the crypt bottom and their progenitors within the transit-amplifying (TA) zone.[16] IESCs were identified as Lgr5^{+} and/or *Ascl2*$^{+}$ crypt base columnar cells (CBC) that asymmetrically divide into one new IESC and one TA cell to maintain intestinal homeostasis, or divide symmetrically into two daughter TA cells only upon tissue expansion or damage.[17] Interestingly, TA daughter cells can hyper-proliferate to replace injured IECs or convert into IESCs during GI injury.[18] The underlying mechanisms by which IESCs and daughter TA cells inter-convert upon GI injury, inflammation, and diseases are largely unclear. It has been recognized that the disturbed homeostasis from IESCs to progenitors causes intestinal neoplasia.[19] In this study, combining mouse injury models with patient-based observations, we investigated the role of CDC42 in the maintenance of IESCs and more rapidly proliferating progenitors during GI injury. Based on our findings, we provide a model of how IESCs respond to GI diseases and injury and suggest a potential target for direct intervention in chronic GI inflammation or inflammation-induced CRC.

Materials and methods

Materials

Details were listed in the **Supplemental Materials and Methods.**

Animal resources and maintenance

The animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at CWRU, Cleveland, OH, USA (IACUC2021-0012), at CCHMC, Cincinnati, USA (IACUC2016-0100), and at ILAS, Beijing, China (HXN16001). Breeding pairs of *Villin-Cre*ER^{T2} and *Cdc42*$^{f/f}$ mice were from CCHMC.[12,20] C57BL/6, *Villin-Cre*, and *Lgr5*-EGFP-IRES-CreER^{T2} transgenic mice were purchased from Jackson Labs (Bar Harbor, MA). Constitutive or inducible depletion of *Cdc42* from IECs was achieved by breeding *Cdc42*$^{f/f}$ mice with *Villin*-Cre or *Villin*-CreER^{T2} transgenic mice.[21] The inducible depletion of *Cdc42* upon Lgr5 or Bmi1 IESCs was investigated by crossing *Cdc42*$^{f/f}$ mice with Lgr5-eGFP-IRES-CreER^{T2} or/and *Villin-Cre*ER^{T2}, or *Bmi1-Cre*ER^{T2}mice (JAX strain, 010,531; Fig. S1A—D).[22] All mice used in these studies have been backcrossed with C57BL/6 for more than ten generations and were re-genotyped with respect to *Cdc42*$^{f/f}$ and Cre prior to necropsy. All studies were performed with littermate *Cdc42*$^{f/f}$ mice designated as wild-type controls. *Cdc42*$^{f/f}$ mice with *Villin*-Cre, *Villin-Cre*ER^{T2}, or Lgr5-eGFP-IRES-CreER^{T2} denoted as *Vil-Cre*;*Cdc42*$^{f/f}$, *VilCre*ER;*Cdc42*$^{f/f}$, or *Lgr5Cre*ER;*Cdc42*$^{f/f}$ mice,[21] were maintained under specific pathogen-free conditions in the CCHMC and ILAS Animal Care Facility.

Biopsies and surgical specimens from IBD and CRC patients and tumor tissue microarray

CRC biopsies from 340 patients were obtained as previously described.[23] Tissue samples originated from specimens of patients who underwent surgical therapy of colorectal carcinoma at UICC stage II or III at the Surgical Department of the Jena University Hospital of Friedrich-Schiller University. All specimens had negative margins. Data on clinical parameters, including sex, age, tumor stage, and follow-up information, were extracted from the prospective tumor registry of the surgical clinic. Pathologic findings (*i.e.*, site of primary tumors, depth of tumor invasion, grading, lymphatic vessel invasion, venous invasion) were obtained from the pathologists' original reports.[24] The CRC Tissue Microarray Assay (TMA) was assembled using 0.6 mm punch biopsies from all 340 samples according to standard procedures and was described before.[23,25]

Colonic surgical samples from adult patients including 19 CD, 23 ileal CD, 17 UC, and 40 diagnosed colorectal adenocarcinoma patients were obtained from the University of Cincinnati Medical Center, Cincinnati, USA, division of pathology, MHMC, Cleveland, USA, and Peking Union Medical College Hospital (PUMC hospital), Beijing, China.

RNA samples of 35 cases of human IBD colonic biopsies or surgical specimens, including 10 cases of diagnosed healthy controls, 18 cases of diagnosed CD patients, and 7 cases of diagnosed UC patients were from a biospecimen bank of Department of Gastroenterology, the Affiliated Hospital of Qingdao University, Qingdao, China. Informed consent was obtained from all of the subjects during the initial enrollment.

Prior to processing for HE, histology, RNA extraction, and patient enrollment, IRB or de-identified IRB protocols for using IBD and CRC patient samples were approved respectively by the ethics committee of Affiliated Hospital of Qingdao University, Qingdao and PUMC Hospital, Beijing, China, the local ethics committee (EC) of Jena General Hospital, Germany, and de-identified Institutional Review Board (IRB) protocols at CCHMC, Cincinnati (XH, IRB2009-1680) and MHMC/CWRU Cleveland (XH, IRB21-00237), OH, USA. All research was performed in accordance with relevant guidelines/regulations using human samples.

Animal resources and maintenance

The animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at CCHMC, Cincinnati, OH, USA (IACUC2016-0100), IACUC at MHMC and CWRU, Cleveland, OH, USA (IACUC 2020—0081), and ILAS, Beijing, China (HXN16001). All mice were maintained under specific pathogen-free conditions in the CCHMC, MHMC/CWRU, and ILAS Animal Care Facility. All experiments were performed in accordance with relevant guidelines and regulations of live vertebrates. Detailed procedures for mouse crossing (Fig. S1A—D) were described in the **Supplemental Methods.**

Radiation-induced injury models

The detailed procedure was described in the **Supplemental Methods.** The mouse numbers in each group are greater than 5 IEC or IESC *Cdc42* deficient mice.

Animal model of colitis

Detailed method was described in the **Supplemental Methods.** The mouse numbers in each group are greater than 5 IEC or IESC *Cdc42* deficient mice.

Immunohistochemistry (IH), immunofluorescence (IF), and immunoblotting for anti-CDC42, β-catenin, Ki67, Lgr5, quantitated real-time PCR (QPCR), and TMA

Detailed methods were described in the **Supplemental Methods.**

Enteroid and colonoid culture

Detailed culturing was described in the **Supplemental Materials and Methods.**

Flow cytometry (FACS)

Detailed method was described in the **Supplemental Methods**.

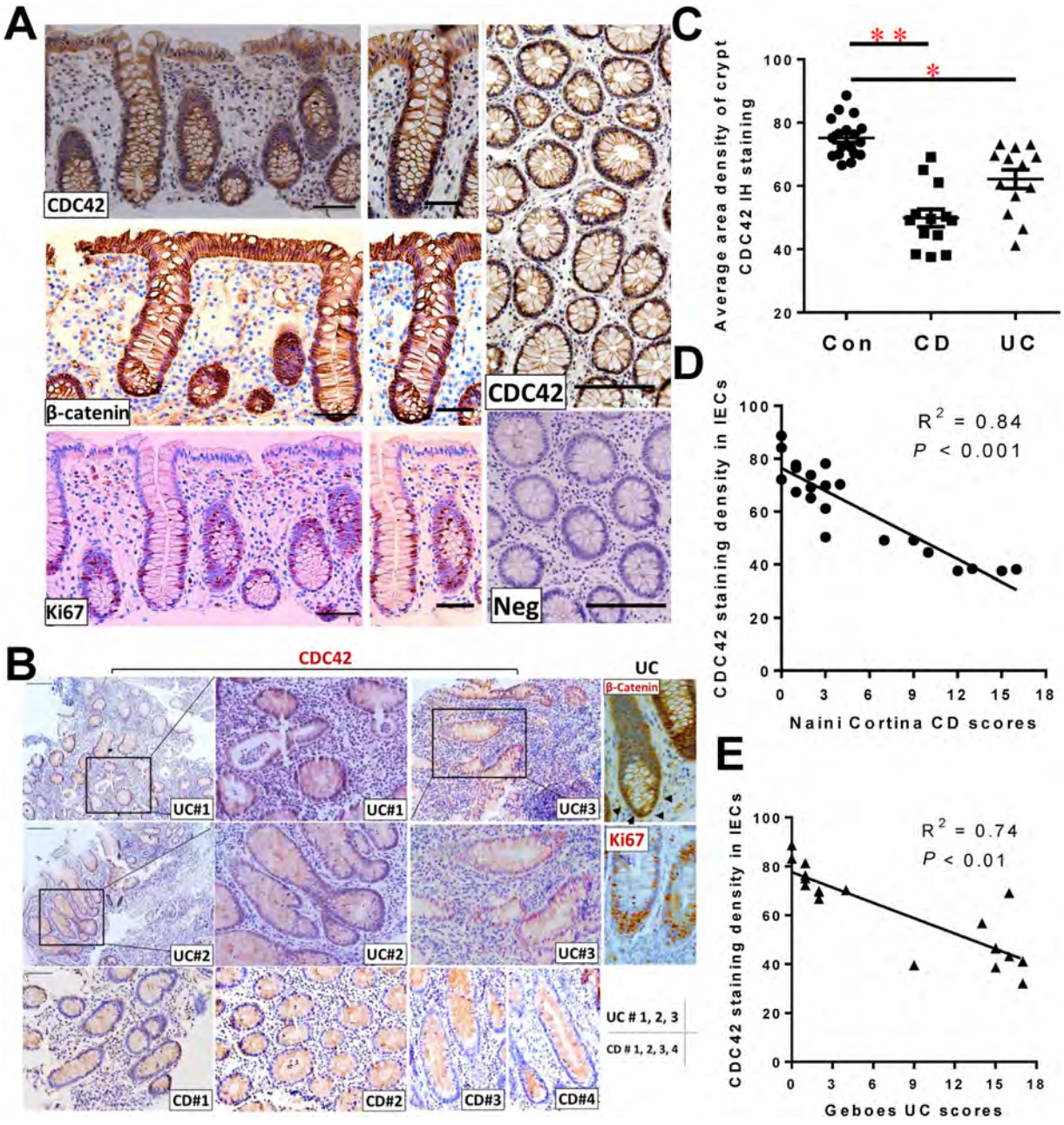

Figure 1 Aberrant expression of CDC42 is correlated with IBD. **(A, B)** CDC42 expression was retrospectively investigated in colonic surgical samples in the adult CD (total 19 cases), ileal CD (23 cases), and UC (17 cases) patients were obtained from the University of Cincinnati Medical Center, Cincinnati, USA, and Peking Union Medical College Hospital (PUMC Hospital), Beijing, China. CDC42, β-catenin, and Ki67 expressions were examined in the colonic IECs of CD and UC samples. The area of magnified images on right is boxed in images on left. "Neg" represents a negative staining control for CDC42 antibodies. **(C)** The area intensity of CDC42 staining in the colonic crypts was quantified in healthy controls, CD, and UC patients. Minimal 200 crypts per group were measured. $n > 5$ patients per group. **(D, E)** Linear regression analysis was used to analyze the correlation between CDC42 IH staining density with histological disease activities in UC and CD. Results are expressed as mean ± SEM (**$P < 0.01$ or *$P < 0.05$ *vs.* healthy controls). Scale bars = 200 μm.

Gamma-secretase inhibitor XX (GSI-20; DBZ; DIBENZAZEPINE (S,S)-2-[2-(3,5-Difluorophenyl)acetylamino]-N-(5-methyl-6-oxo-6,7-dihydro-5H-dibenzo [b,d]azepin-7-yl) propionamide) **treatment.**

Detailed method was described in the **Supplemental Methods.**

Statistical analysis

For all data compilation, the statistics software package SPSS (version 19.0) or GraphPad Prism (6.0) was used. Univariate survival analysis was subsequently carried out separately for each investigated parameter using

Kaplan—Meier estimates. Survival curves were compared and assessed using the log-rank test. *P* values of 0.05 or less were considered significant when using *t*-tests and analysis of variance (ANOVA). All statistics for colonic tissue arrays were accredited by a biostatistician of the Institute of Medical Statistics, Computer Sciences, and Documentation, Jena University Hospital.

Results

Aberrant expression of CDC42 is correlated with IBD

CDC42 is highly expressed in GI epithelia (Fig. 1A; Fig. S2A—C), and loss of CDC42 in the GI tract induces defective IEC proliferation and migration.[12] However, it is not clear if aberrant CDC42 expression regulates the GI disease course in humans or whether targeting CDC42 represents an option in gut disease. To test this hypothesis, we retrospectively investigated CDC42 expression in the colonic surgical samples obtained from adult CD (19 cases), ileal CD 23 cases), and UC (17 cases) patients obtained at the University of Cincinnati Medical Center, Cincinnati, USA, and Peking Union Medical College Hospital (PUMC Hospital), Beijing, China. CDC42 immunohistochemistry (IH) staining (Fig. 1A and B) combined with semi-quantitated pathology along with stringent negative controls showed that CDC42 expression was markedly reduced in the colonic IECs of CD samples and UC samples compared to normal patients (Fig. 1C). Interestingly, Ki67$^+$ and β-Catenin$^+$ crypt IECs are increased in UC samples (Fig. 1A, B). Linear regression analysis revealed that reduction in the crypt CDC42 was negatively correlated with histological disease activities in UC and CD that were determined by Geboes and global histological activity (GHA) scores,[26] respectively (Fig. 1D, E). To confirm the transcriptional effects of *CDC42* on IECs in IBD, we enrolled healthy controls (10), CD (18), and UC (7) patients with informed consent at Qingdao University, Qingdao, China, and then performed quantitated real-time PCR (QPCR) with endoscopic biopsies. Consistently, QPCR results showed a significant reduction of *CDC42* gene expression in CD and a trend toward reduction of *CDC42* expression in UC (Fig. S2A). These data indicated that the reduction of crypt *CDC42* expression might be associated with mucosal chronic inflammation-induced IBDs although more samples or laser capture microdissection (LCM)-captured crypts used for QPCR analysis might be required for future research.

Aberrant expression of CDC42 is associated with CRC pathogenesis

Aberrant expression or activity of CDC42 has been reported in CRC.[13] To test the association of CDC42 with colonic IESCs, we utilized β-catenin, Ki67, and Lgr5 IH and CDC42 Tissue Microarray Assay (TMA) to retrospectively study the CDC42 in the Lgr5$^+$, β-catenin$^+$, or Ki67$^+$ colonic cancer stem cells (CSCs) from patients diagnosed with CRC. Similar to the distribution of β-catenin in normal colonic IECs or IESCs, CDC42 was mainly located at the basolateral membranes of IECs and partially co-localized with Ki67$^+$ and Lgr5$^+$ colonic IESCs (Fig. 1A). To study CDC42 expression in CRC without therapeutic interventions, we collaborated with the Jena University Hospital of the Friedrich Schiller University in Jena to obtain 340 CRC specimens dating from 1993 to 2006. These patients had undergone colectomies only to remove the cancerous colonic tissues. Surgical pathologists categorized the majority of these CRC tissues as having low CDC42 expression. Histological diagnosis was established according to the guidelines of the World Health Organization.[23,25] Based on CDC42 immunochemistry staining (Fig. 2A), the 340 samples were denoted as "0" for histologically normal (0.9%, $n = 3$), "1" for mild cases (17.0%, $n = 58$), "2" for moderate (55.6%, $n = 189$) and "3" for severe (26.5%, $n = 90$) cases. TMA revealed that CDC42 expression was positively correlated with CRC disease severity (Fig. 2B). More importantly, the stronger CDC42 staining, including scores "2" and "3", had a significant reduction in overall patient survival over the time course of five years (Fig. 2C). These data indicate that highly expressed CDC42 could be a negative prognostic marker for CRC progression and may potentially be used to determine if patients require aggressive surgery or other treatments. To test the possibility that CDC42 levels could be used as a tool for determining CRC treatment, we studied surgical specimens provided by the University of Cincinnati General Hospital in Cincinnati, PUMC Hospital in Beijing, and MHMC in Cleveland, from patients that had undergone either radiation- or chemo-therapies prior to surgical procedures. Concomitant with observations made at the Jena University Hospital, these post-treatment CRC samples showed increased CDC42, β-catenin, Ki67, and Lgr5 expression in the CRC area, and CDC42 staining was partially co-localized with Ki67$^+$ or Lgr5$^+$ colonic CSCs (Fig. 2D). Strikingly, we observed negligible CDC42 staining in the central cancerous areas along with less Ki67 and β-catenin nuclear abundance (Fig. 2D), which were reported as more differentiated cancerous cells.[27] In contrast, stronger CDC42 staining was found in the adjacent normal area of proliferative crypts (Fig. 2D), suggesting that CDC42 might be involved in the CRC invasive behaviors.[28] Our data demonstrate that aberrant expression or activity of CDC42 is associated with human GI inflammation and CRC pathogenesis. However, considering that studying the expression change in the IEC CDC42 upon treatments will need a large cohort of CRC patients, our results only provide the implication of the role of CDC42 in CRC treatment outcomes. Taken together, our data suggest that intestinal CDC42 expression could be used as a potential marker for GI chronic inflammation and implicate that CDC42 inhibition might enhance the effectiveness of CRC treatments.

CDC42 co-localizes with Lgr5$^+$ IESCs at crypt bases

We then detected the expression of CDC42 in the mouse intestine and colon. CDC42 IH showed that it was expressed throughout intestinal and colonic IECs but was confined to the crypts including IESCs and progenitors in the TA zone (Fig. S3A, B). In *Lgr5*-eGFP-IRES-CreER^{T2} (*Lgr5*CreER) mice,

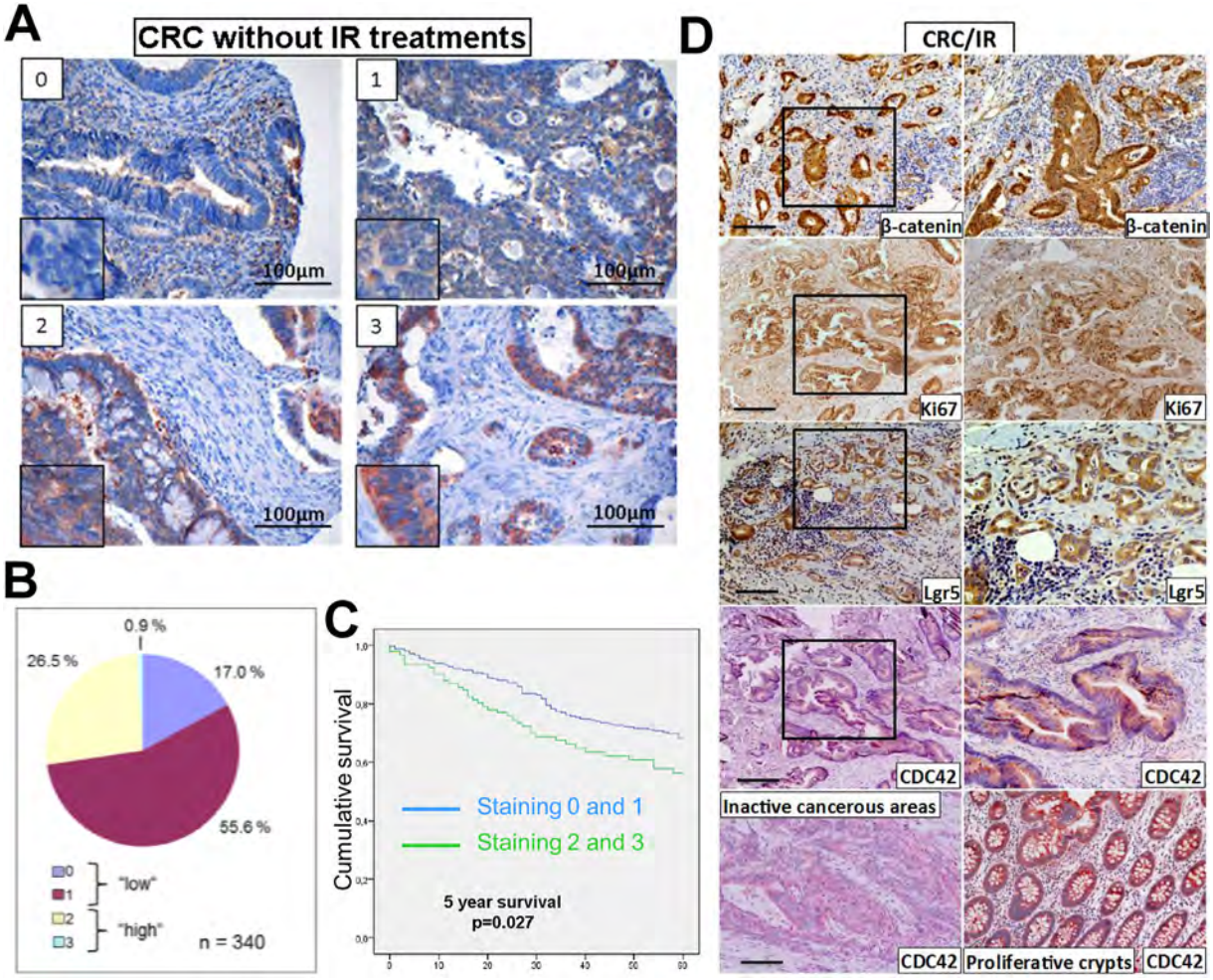

Figure 2 Aberrant expression of CDC42 in the CRCs. **(A, B)** CDC42 levels were determined by IH in tissues surgically resected from 340 European CRC patients obtained between 1993 and 2006. Surgical pathologists categorized all CRC patients as either low CDC42 expression including histologically normal denoted as "0" and mild denoted as "1", or high CDC42 expression including moderate denoted as "2" and 0.9% severe denoted as "3" **(C)** Patients were followed up for an overall 5 years after colectomy to determine survival, and the correlation with CDC42 staining intensity was determined. **(D)** CDC42 IH with surgical specimens from the University of Cincinnati Medical Center and PUMC Hospital. Representative images display β-catenin, Lgr5, Ki67, and CDC42 IH staining in the cancerous area, inactive cancerous areas, and proliferative crypts in CRC tissues. The area of magnified images on right is boxed in images on left. Scale bars = 200 μm.

a Lgr5^{+} IESC reporter mouse line, CDC42 immunofluorescence staining (IF) showed that the protein partially co-localized with the majority of Lgr5^{+} IECSs at the bases of the small intestinal and colonic crypts (Fig. S3A, B). Low levels of CDC42 were also found within surface IECs in the mouse intestine and colon stained by CDC42 IH. We then stained human CDC42 in surgical intestinal and colonic tissues. Similarly, in human intestinal and colonic tissues, we found that CDC42 was expressed in IESCs and TA IECs located away from the surface IECs (Fig. 1A; Fig. S2B, 3C, 3D). Collectively, these observations suggest that CDC42 is expressed in IESCs, and may play a role in IESC/progenitor in both the intestine and colon. We next tested the effects of monogenic depletion of *Cdc42* or pharmacological inhibition of CDC42 upon murine IECs.

Constitutive depletion of intestinal *Cdc42* leads to a prolonged mucosal inflammation that induces crypt dysplasia

The intestinal villus-crypt architecture is dynamically maintained by differentiated IECs, undifferentiated IESCs residing at the bottom of crypts, and differentiating progenitors in the TA zone.[16] Impaired homeostasis of IESCs and progenitors leads to hyperplastic IEC, altered villus-crypt architecture and mucosal inflammation, and further crypt dysplasia.[29] To study the role of CDC42 in mucosal inflammation and dysplasia, we first crossed *Cdc42* floxed mice (*Cdc42*$^{f/f}$) with *Villin*-Cre mice (called *Vil*Cre;*Cdc42*$^{f/f}$ hereinafter; Fig. 3A), by which *Cdc42* is constitutively deleted in

all IECs, including active or quiescent IESCs and their progenitors[12,30] (Fig. 3A). Intestinal morphology was quantified by villus length, villus width, and crypt depth, and mucosal inflammation in the 1-, 3-, 4- and 6-month-old mice. Compared to littermate controls (*Cdc42*$^{f/f}$), *VilCre*;*Cdc42*$^{f/f}$ mice exhibited a disrupted villus-crypt architecture and mucosal inflammation, characterized by a significant increase in the crypt depth, villus width, and villus length and inflammatory cell infiltration (Fig. 3B, C, H). Furthermore, *VilCre*;*Cdc42*$^{f/f}$ mice displayed crypt dysplasia (crypt→villus hypertrophy, IEC hypertrophy and multilayers, villus fissions and "cyst" structures) (Fig. 3B, C). These morphological aberrations in the small intestines (intestine) were further progressed in the 4- and 6-month-aged mice compared to 1-month-old *Cdc42* depleted mice ($n \geq 5$ per group, $^*P < 0.01$ *vs. Cdc42*$^{f/f}$; Fig. 3C). Interestingly, villus length in the 6-month-old *VilCre*;*Cdc42*$^{f/f}$ mice was significantly shorter than 1-month-old *VilCre*;*Cdc42*$^{f/f}$ mice in contrast to significantly increased mucosal inflammatory cells, crypt depth and width (Fig. 3C), indicative of mucosal age-associated inflammation. These data suggest that CDC42 is required for the intestinal crypt→villus architecture and barrier formation that is maintained by the transition from IESCs to progenitors (IESCs→progenitor). Loss of CDC42 in IECs accumulates mucosal chronic inflammation, leading to crypt dysplasia.

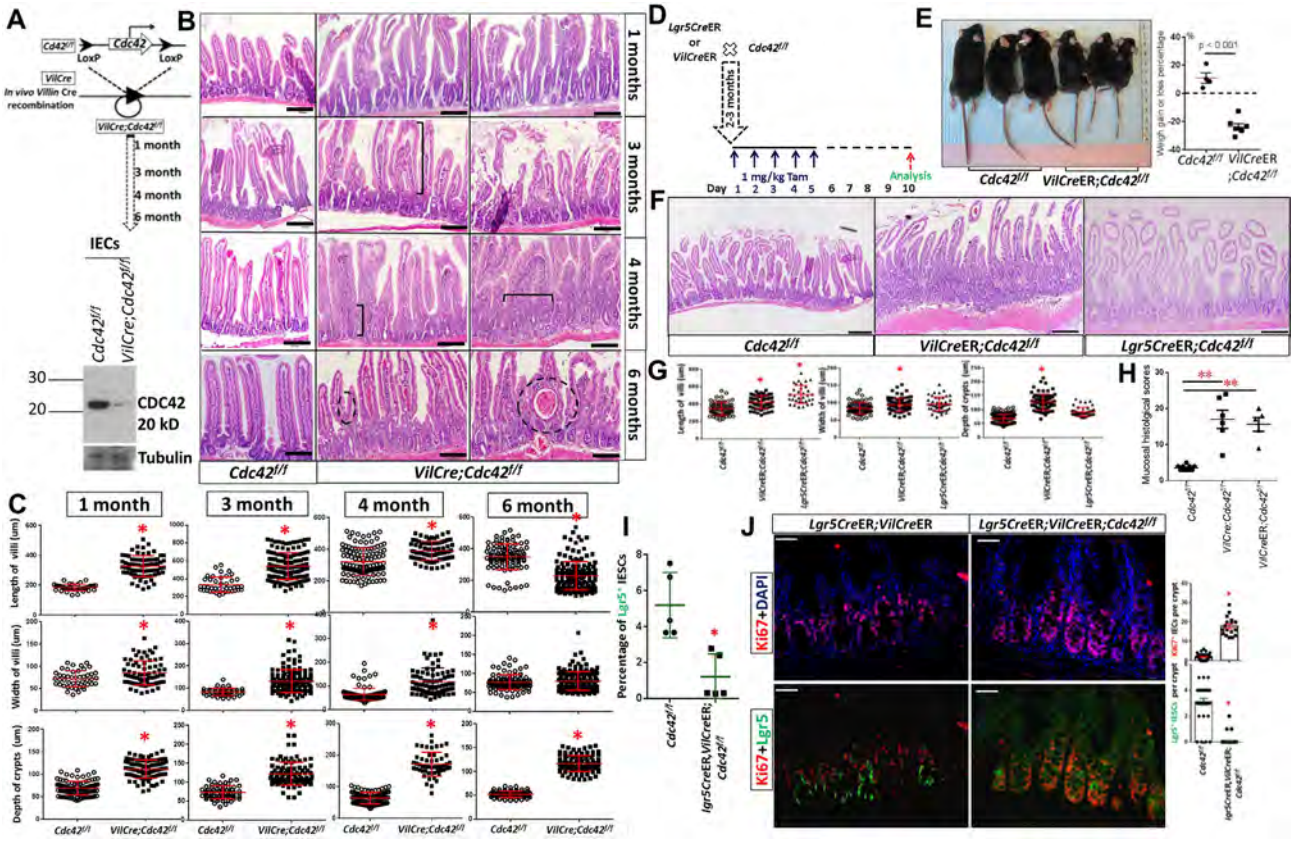

Figure 3 Constitutive depletion of intestinal *Cdc42* leads to a prolonged mucosal inflammation that induces crypt dysplasia. **(A)** *Cdc42* floxed mice were crossed with *VilCre* mice to persistently delete *Cdc42* in IECs. *Cdc42* depletion was confirmed by immunoblotting of total proteins isolated from the IECs. **(B)** Mucosal histology was examined in 1-, 3-, 4-, and 6-month-old *VilCre*;*Cdc42*$^{f/f}$ mice ($n \geq 5$ mice per group). Brackets denote villus length, villus width, and crypt depth. Unfilled circles represent villus fission and cyst structure. **(C)** Quantification of length and width of villi, and crypt depth in 1-, 3-, 4-, and 6-month-old *VilCre*;*Cdc42*$^{f/f}$ mice. Results are expressed as the mean ± SEM ($n \geq 5$ mice per group, $P \leq 0.01$). Scale bars = 200 μm **(D)** *Vil*CreER;*Cdc42*$^{f/f}$ and *Lgr5*CreER;*Cdc42*$^{f/f}$ mice were given 1 mg/kg tamoxifen (Tam) intraperitoneally for five days, and then Cre-LoxP recombination for another five days to inducibly delete *Cdc42* from IECs or IESCs. **(E)** *Vil*CreER;*Cdc42*$^{f/f}$ mice lost a significant amount of weight following Tam treatment. **(F, G)** Villus width, villus length, and crypt depth were measured in HE-stained intestines. Results are expressed as the mean ± SEM ($n \geq 5$ mice per group, $^*P \leq 0.01$ *vs. Cdc42*$^{f/f}$). **(H)** Intestinal mucosal inflammation including IEC hyperplasia (0–3), crypt hypertrophy (0–3), crypt elongation (0–3), immune cell infiltration (0–3), and mucosal thickness, was scored in the Cdc42$^{f/f}$, *VilCre*;Cdc42$^{f/f}$ (B) and *VilCreER*;Cdc42$^{f/f}$ mice (F). **(I)** Lgr5 low, mid, and high populations of IESCs were analyzed by FACS. Lgr5^{hi} populations were significantly lower in *Lgr5*CreER;*Vil*CreER;*Cdc42*$^{f/f}$ mice. Results are expressed as the mean ± SEM ($n \geq 5$ mice per group, $^*P \leq 0.01$ *vs. Cdc42*$^{f/f}$). **(J)** Intestinal tissues were isolated from *Lgr5*CreER;*VilCre*ER or *Lgr5Cre*ER;*VilCre*ER;*Cdc42*$^{f/f}$ mice and double stained for Ki67 (red) and/or Lgr5 (green) by IF. The number of Ki67$^+$ IECs and Lgr5$^+$ IESCs per crypt was counted in 200 crypts. Results are expressed as the mean ± SEM ($n \geq 5$ per group, $^*P \leq 0.01$). Scale bars = 200 μm.

Inducible depletion of intestinal *Cdc42* reduces Lgr5$^+$ IESC numbers and increases IEC progenitor hyperplasia and mucosal inflammation, resulting in crypt dysplasia

To assess the direct role of IEC CDC42 in the IESCs→progenitor and mucosal inflammation, we crossed *Cdc42*$^{f/f}$ with *Vil*Cre$^{ER^{T2}}$ mice (called *Vil*CreER;*Cdc42*$^{f/f}$ hereinafter). In these *Vil*CreER;*Cdc42*$^{f/f}$ mice, depletion of *Cdc42* from IECs was induced by 5 days of tamoxifen (Tam) administration (Fig. 3D; Fig. S1B). Inducible IEC-*Cdc42* deficient mice had significantly reduced weight gain compared to controls after 5-day Tam induction and Cre recombinase (Fig. 3E). These mice also exhibited increased villus width, villus length, and crypt depth and mucosal inflammation (Fig. 3F–H) but did not develop intestinal "cyst" structures (Fig. 3F, G). We next crossed *VilCre*;*Cdc42*$^{f/f}$ mice with *Lgr5Cre*ER mice (called *Lgr5*CreER;*Vil*CreER;*Cdc42*$^{f/f}$ hereinafter) to study the effects of intestinal *Cdc42* depletion on Lgr5$^+$ IESC numbers, proliferation, and localization that affect IEC barrier formation.[31] *Lgr5Cre*ER;*VilCre*ER;*Cdc42*$^{f/f}$ mice exhibited a disrupted villus-crypt architecture similar to *VilCre* or *VilCre*ER;*Cdc42*$^{f/f}$ mice (Fig. 3B, F; Fig. S4A). Consistent with previous reports,[12,15] using GFP and lysozyme (Lyso) IF, we also found that Lgr5$^+$ IESCs and Paneth cells were mislocalized from intestinal crypts to villi in the *Lgr5*CreER;*Vil*CreER;*Cdc42*$^{f/f}$ mice (Fig. S4A, C). Bmi1, a member of polycomb-repressing complex 1 (PRC1), can mark a population of quiescent IESCs that repopulates Lgr5$^+$ IESCs during injury.[32,33] To observe the role of CDC42 on other IESCs, we crossed *Cdc42*$^{f/f}$ with *Bmi1Cre*$^{ER^{T2}}$ mice to inducibly delete CDC42 in Bmi1$^+$ IESCs. Compared with *VilCre*ER;*Cdc42*$^{f/f}$ mice, *Cdc42* depletion in Bmi1$^+$ cells led to intestinal clustered dysplastic crypts and mild mucosal inflammation (Fig. S4B). Ki67:Lgr5-GFP double staining and fluorescence-activated cell sorting (FACS) revealed that inducible depletion of IEC *Cdc42* led to markedly reduced Lgr5$^+$ IESCs at the crypt bases in *VilCre*ER;*Cdc42*$^{f/f}$ mice (Fig. 3I, J) in contrast to increased Ki67$^+$ IEC progenitor numbers and proliferation (Fig. 3J). These data together with histopathological analyses (Fig. 3H) suggest that progenitors undergo hyperplasia and mucosal inflammation. Taken together, our data demonstrate that loss of *Cdc42* in the different populations of IESCs disrupts Lgr5$^+$ IESC/progenitor homeostasis, resulting in crypt dysplasia and mucosal inflammation. CDC42 maintains the normal morphology of intestines, in part by controlling active or quiescent IESC→progenitor homeostatic proliferation.

Inducible depletion of intestinal *Cdc42* increases γ-irradiation-induced injury

Irradiation leads to specific injury to CBCs, which can induce enteritis and colitis.[34] After exposure to a high dose of irradiation, cycling CBCs undergo DNA double-strand break followed by rapid apoptosis or mitotic death, resulting in lost proliferative or regenerative capacity.[31,34] Depending on the irradiation dosage, progenitor pools or other IESCs replenish lost CBCs, for example, Bmi1$^+$ IESCs are resistant to irradiation injury and can be activated followed by damage to the active IESCs.[18,33] Thus, irradiation is often utilized to test IESC/progenitor proliferative or regenerative function. To determine the role of CDC42 in the intestinal IESC/progenitor activity and IESC/progenitor function after injury, either *VilCre* or Tam-inducible *VilCre*ER recombination was used to constitutively or inducibly delete *Cdc42* in IECs. *Vil*Cre and *VilCre*ER;*Cdc42*$^{f/f}$ mice were then irradiated (Fig. 4A). Constitutive depletion of *Cdc42* in IECs significantly reduced the number of regenerated crypts 3 days post-irradiation in comparison with littermate controls (Fig. 4B). Consistently, inducible depletion of *Cdc42* showed significantly reduced the number of regenerated crypts compared to controls (0.6 ± 0.1 crypts per mm in *VilCre*ER;*Cdc42*$^{f/f}$ versus 13.5 ± 0.5 per mm in *Cdc42*$^{f/f}$ mice, $n = 3$, $^*P < 0.01$; Fig. 4B, C). Both *VilCre*;*Cdc42*$^{f/f}$ and *VilCre*ER;*Cdc42*$^{f/f}$ mice exhibited significantly worse mucosal injury post-irradiation (Fig. 4B, C). These data indicate that CDC42 is required for IESC/progenitor regeneration; in turn, loss of CDC42 deteriorates mucosal healing. By crossing *Lgr5Cre*ER with *Cdc42*$^{f/f}$ mice, *Cdc42* can be depleted in IESCs in a mosaic fashion.[35] Interestingly, we found that depletion of *Cdc42* led to reduced bromodeoxyuridine (BrdU) incorporation into IECs in the regenerated crypts following irradiation.

Paneth cells are located at the bottom of crypts, playing a niche role in protecting IESC self-renewal and proliferation. Loss of Paneth cell niches leads to reduced Lgr5$^+$ IESC numbers.[36] Intriguingly, Paneth cell precursors are postulated to represent dormant IESCs that can be reactivated after injury to become Lgr5$^+$ IESCs.[37] Previous work has demonstrated that CDC42 controls Paneth cell migration and maturation.[15] Consistently, utilizing 12-Gy irradiation injury, we found that inducible depletion of *Cdc42* caused significantly increased Paneth cell migration and reduced numbers of niche Paneth cells at the regenerated crypts (Fig. 4D–F). Similarly, *LgrCre*ER;*Cdc42*$^{f/f}$ mice exhibited significant Paneth cell migration to the villus surface in the irradiation-treated mice compared to control mice (Fig. 4D, F; Fig. S4C), but did not show reduced numbers of Paneth cells at the crypt base (Fig. 4D, E). In addition, dysfunctional or misallocated Paneth cells can be the site of origin for intestinal inflammation.[38] Therefore, our also data suggest that IEC CDC42 might have a role in maintaining the Paneth cell niche. Loss of *Cdc42* could impair Paneth cell maturation to control mucosal inflammation.

Depletion of colonic *Cdc42* leads to transformed colonic IECs sensitive to irradiation-induced colitis

Aging is considered to be a GI stressor and is directly associated with senescence-associated mucosal inflammation and IEC transformation.[29,39] In addition, CDC42 has been reported to suppress the metastasis of intestinal tumors and mutant *Cdc42* has been identified as a risk factor for colorectal cancer.[40] To determine if loss of *Cdc42* is sufficient to alter IEC transformation in aged mice, we examined colon morphology in 9- to 13-month-old *VilCre*;*Cdc42*$^{f/f}$ mice devoid of CDC42 (Fig. 5A). We found that persistent depletion of *Cdc42* in the colon led to colonic IEC hyperplasia, mucosal inflammation, and sporadic

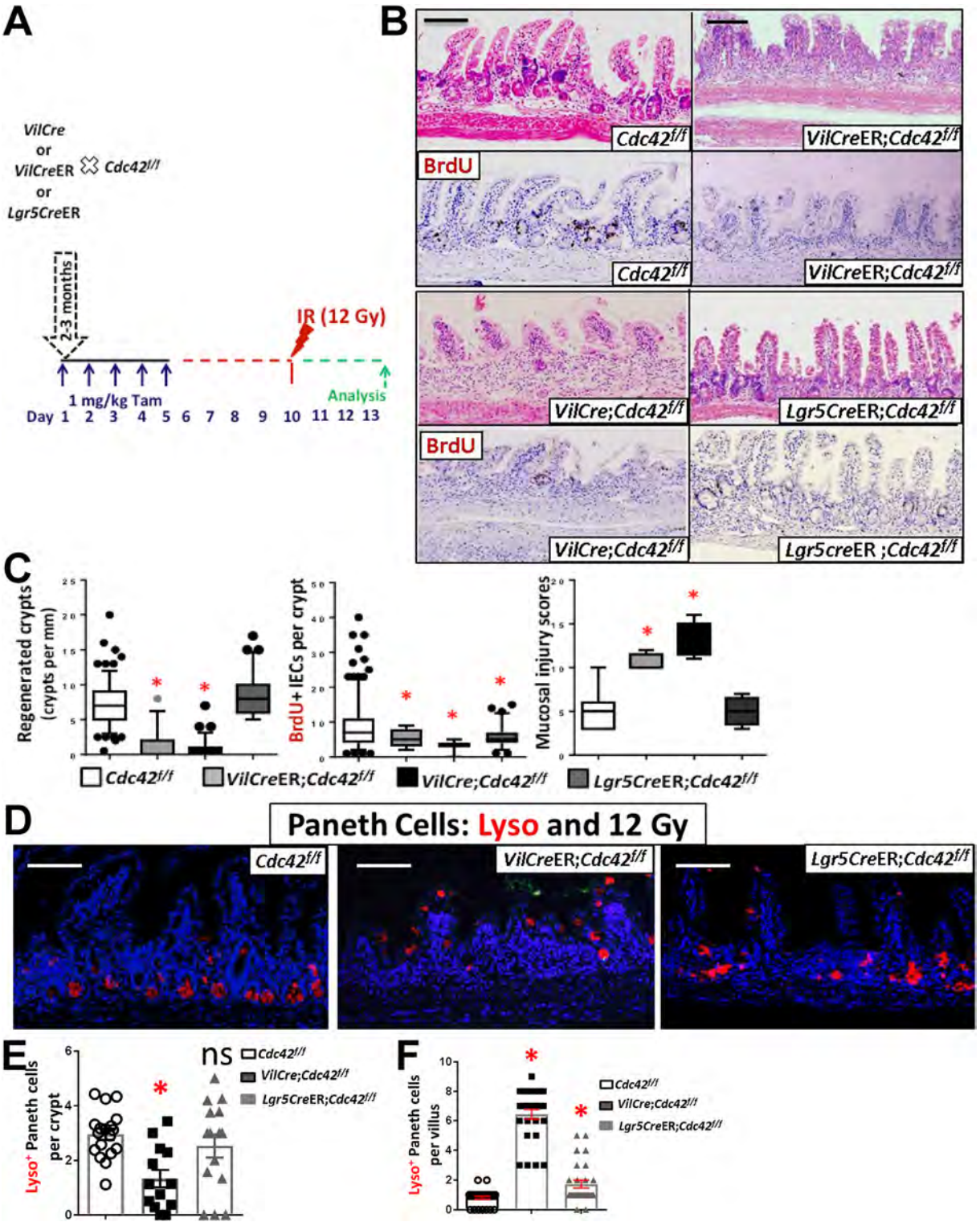

Figure 4 Inducible depletion of intestinal *Cdc42* increases γ-irradiation-induced injury. **(A)** *Vil*CreER;*Cdc42*$^{f/f}$ and *Lgr5*CreER;*Cdc42*$^{f/f}$ mice were given 1 mg/kg Tam for five days, 10 min of 12 Gy irradiation following five-day Cre recombination after the last Tam injection, and then sacrificed three days post-irradiation **(B, C)** Crypt regeneration was quantified by counting regenerated mucosal crypts and BrdU staining. The intestinal mucosal injury was determined by histological scores. Results are expressed as the mean ± SEM ($n = 6$ or 7 mice per group, $^*P \leq 0.01$). **(D—F)** Paneth cells were identified by lysozyme (Lyso) IF. Lyso$^+$ Paneth cells were counted within crypts and on the surface of villi. Results are expressed as the mean ± SEM ($n = 6$—7 mice per group, a total of 200 crypts per group, $^*P \leq 0.01$). Scale bars = 200 μm.

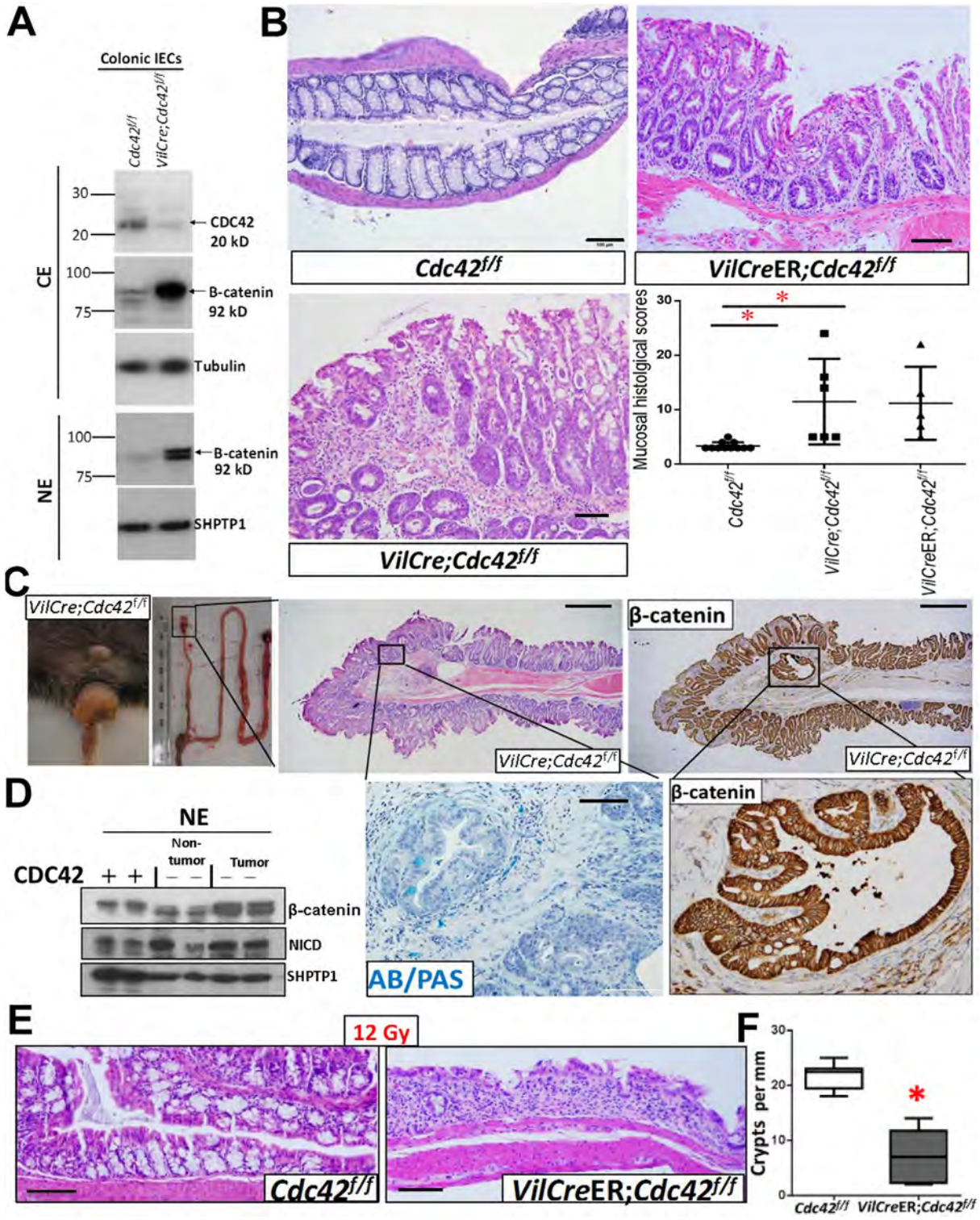

Figure 5 Depletion of colonic *Cdc42* leads to transformed colonic IECs sensitive to irradiation-induced colitis. **(A)** Colonic IECs were isolated for extracting cytosolic and nuclear proteins (CE and NE). CDC42 expression was determined in CE and β-catenin was determined in NE by immunoblotting. $n = 3$ mice per group. **(B)** Colonic histology in 5-month-old *VilCre*;*Cdc42*$^{f/f}$, *VilCre*ER; *Cdc42*$^{f/f}$, and *Lgr5Cre*ER;*Cdc42*$^{f/f}$ mice were determined by HE staining. The number of aberrant crypt foci (ACF) was counted. Colonic mucosal inflammation including IEC hyperplasia, crypt hypertrophy, crypt elongation, immune cell infiltration, mucosal thickness, ACF, mucin-depleted foci (MDF), was scored in the *Cdc42*$^{f/f}$, *Vil*Cre;*Cdc42*$^{f/f}$ and *Vil*CreER;*Cdc42*$^{f/f}$ mice. Results are expressed as the mean ± SEM ($n \geq 5$ mice per group, $^*P \leq 0.05$ *vs.* *Cdc42*$^{f/f}$) **(C)** Gross lesions were shown in the colorectal area, MDF was shown by Alcian Blue/Periodic Acid Schiff (AB/PAS) staining, and β-catenin was detected by IH. Areas of magnified images underneath are boxed in images. $n = 6–7$ mice per group. **(D)** β-catenin expression was detected in the NE of the colon by immunoblotting. **(E, F)** *VilCre*ER;*Cdc42*$^{f/f}$ mice were exposed to 12 Gy irradiation. Colonic histology (E) and regenerated crypts (F) were quantified 3 days post-irradiation ($n \geq 5$, $^*P \leq 0.01$). Scale bars = 100 μm.

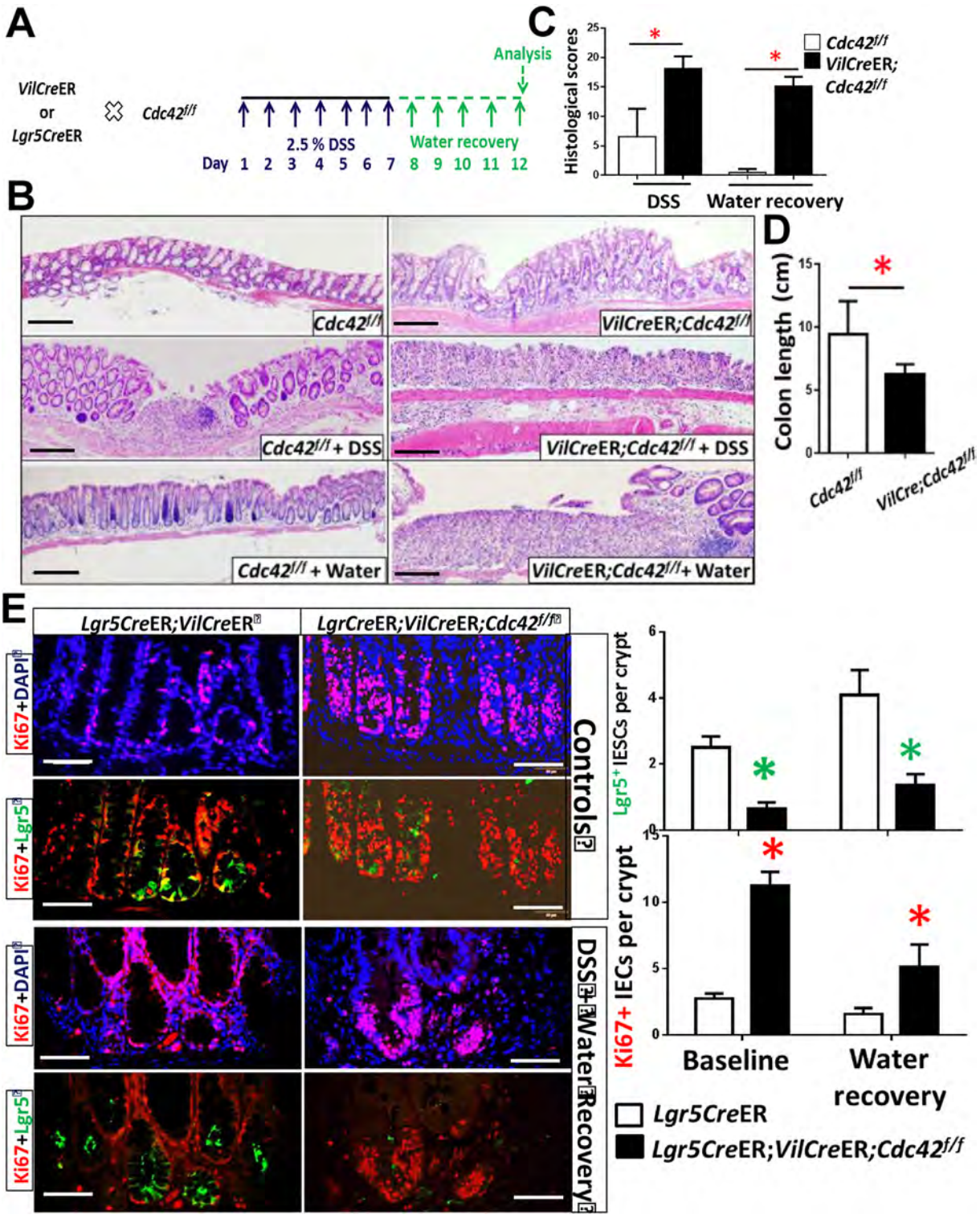

Figure 6 Depletion of *Cdc42* reduces colonic IESC repair, resulting in the susceptibility to colitis. **(A)** After five days of Tam-induced Cre recombination, *VilCre*ER;*Cdc42*$^{f/f}$ and *Lgr5Cre*ER;*Cdc42*$^{f/f}$ mice were exposed to 2.5% DSS in the water for seven days or five days of DSS plus water recovery for five days **(B—D)** Colonic mucosal histology and colon length were quantified after DSS treatment for seven days ($n \geq 5$ mice per group, $^*P \leq 0.01$) **(E)** *Lgr5Cre*ER;*VilCre*ER;*Cdc42*$^{f/f}$ mice were treated for seven days with DSS followed by five days of water recovery. Ki67$^+$ (red) or Lgr5$^+$ (green) IECs were identified and semi-quantified. Results are expressed as the mean ± SEM ($n \geq 5$ mice per group, $^*P \leq 0.01$). Scale bars = 200 μm.

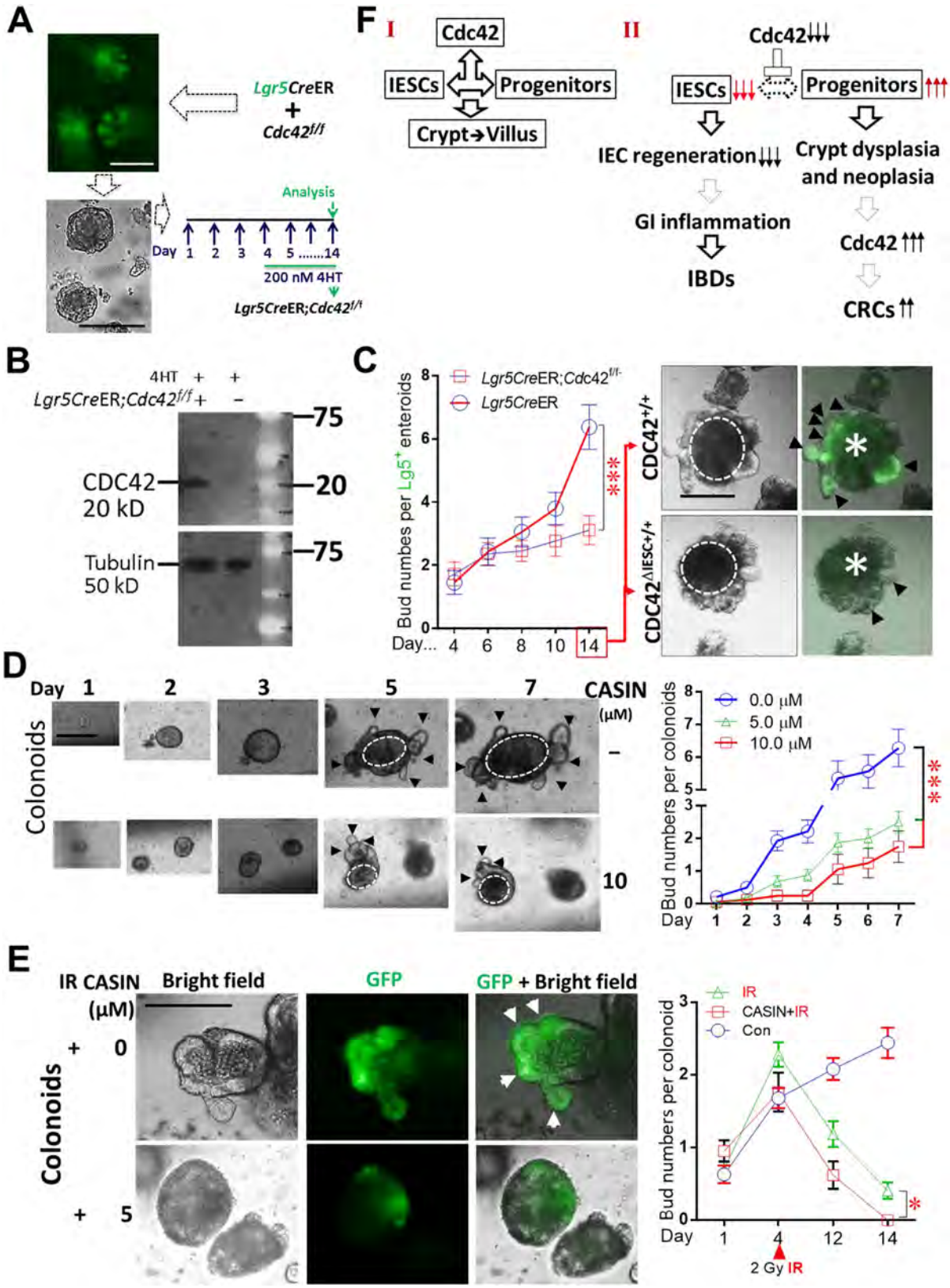

Figure 7 Genetic depletion or pharmacological inhibition of CDC42 sensitized IESCs to injury. **(A)** Intestinal crypts were isolated from *Lgr5Cre*ER;*Cdc42*$^{f/f}$ mice, and resuspended in Matrigel with epidermal growth factor (EGF), Noggin, and R-spondin for culture from day 1 through day 4. *Cdc42* depletion was induced by 200 nM 4HT in the enteroids or Lgr5^{+} enteroids. Enteroids were cultured in six parallel wells per mouse for each experiment (n = 4 mice per group). The number of crypt buds was counted daily in a minimum of 10 enteroids per well after 4HT-induced *Cdc42* depletion. **(B)** Immunoblotting showed the efficiency of CDC42

macroadenoma displaying colorectal polyps, distorted or serrated crypts, mucin-depleted foci (Fig. 5B, C; Fig. S5A). Importantly, persistent depletion of *Cdc42* in IECs led to colorectal adenoma, which was displayed in 7 out of 9 mice and characterized by aberrant crypt foci (ACF) and clustered mucin-depleted foci (MDF), strongly indicating the role of Cdc42 in the intestinal neoplasia (Fig. 5C, inserts). Immunoblotting showed that β-catenin nuclear abundance was increased in the crypt in the tumor area compared to the normal colonic or intestinal mucosa in the mutant mice, in which β-catenin was confined mainly between cell boundaries (Fig. 5A, C, D; Fig. S5B). The increased nuclear abundance of β-catenin in the dysplastic crypt IECs might be upon $Lgr5^+$ cell loss in the context of aging. Interestingly, colonic NICD1 expression, which was used to indicate the activation of intestinal Notch signaling,[41] was not different between the colonic normal area and tumor area (Fig. 5D). Consistently, inducible depletion of *Cdc42* in colonic IECs displayed microadenoma with ACF and acute inflammation with severe inflammatory cell infiltration (Fig. 5B and Supplementary Materials, arrows in Fig. S5A). We next exposed inducible IEC *Cdc42*-deficient mice to irradiation injury and examined the regeneration of $Cdc42^{-/-}$ IESCs. We found the limited regeneration of nascent crypts in the colon 3 days post-irradiation and severe IR-induced colitis (Fig. 5E, F), strongly suggesting that loss of *Cdc42* in IECs reduced IESC regeneration and colonic IEC repair, further increased accumulation of mutation. These data indicate that mutant *Cdc42* plays an important role in colonic IEC transformation in the aged colon due to accumulated mucosal inflammation, and susceptibility to injury. Taken together, loss of CDC42 might accumulate colonic IEC mutations to induce spontaneous intestinal inflammation, which causes colitis and high-grade crypt dysplasia. We next investigated the requirement of CDC42 against colonic inflammation.

Depletion of *Cdc42* reduces colonic IESC repair, resulting in the susceptibility to colitis

To study the requirement of CDC42 for controlling colonic inflammation to develop as colitis, IEC CDC42 was inducibly deleted in mice and were then challenged with 2.5% dextran sulfate sodium (DSS) for seven days, which is known to induce colitis in mice[30] (Fig. 6A; Fig. S1B). Following 7 days of DSS treatment, the colitis was more severe in the inducible *Cdc42*-deficient mice than in controls, which is consistent with the above observations that lack of CDC42 leads to irradiation colitis. To determine if the colitis could be healed, we allowed five days of recovery after DSS treatment. Following this recovery period, *Cdc42*-deficient mice displayed increased mucosal injury and shorter colon length compared to controls (Fig. 6B–D), indicating that *Cdc42*-deficient colonic IESCs can reduce the mucosal regenerative and healing capacity. To further test the effects of CDC42 upon colonic IESC regeneration, we exposed *Lgr5Cre*ER;*VilCre*ER;$Cdc42^{f/f}$ mice to the colitis induction and healing paradigm. We found reduced numbers of $Lgr5^+$ colonic IESCs in the *Cdc42*-deficient mice and delayed $Lgr5^+$ IESC recovery following DSS treatment (Fig. 6E). Conversely, colonic progenitors and IECs displayed a remarkable hyperplastic proliferation in *Cdc42*-deficient IECs compared to controls, as indicated by Ki67 staining (Fig. 6E). Together, these data suggest that CDC42 is required for colonic $Lgr5^+$ IESC regeneration. Loss of CDC42 leads to reduced regenerative capacity (IESC → progenitor) that impairs mucosal healing and induces colitis, indicating that CDC42 is a protective factor against colonic injury. Our results also suggest that the dysplastic crypt lesions are secondary to colonic chronic inflammation induced by lack of IEC *Cdc42*.

Inducible depletion or pharmacological inhibition of CDC42 leads to impaired IESC capacity for crypt formation and sensitizes IESCs to irradiation injury

An intact crypt or single $Lgr5^+$ IESC can generate ever-growing, self-organizing "mini-guts" with a defined crypt–villus axis,[42] which is a great *in vitro* model to study the regenerative capacity of IESCs and progenitors after injury or crypt formation capacity during various insults.[43] We isolated and cultured IESCs from *Lgr5Cre*ER;$Cdc42^{f/f}$ or *VilCre*ER;$Cdc42^{f/f}$ mice (Fig. 7A). By adding 4-hydroxy-tamoxifen (4HT) to culture media, *Cdc42* was inducibly deleted in $Lgr5^+$ IESCs[44] (Fig. 7B). Lgr5-GFP^+ crypts and crypt formation of enteroids were used to evaluate the effects of CDC42 upon the IESC proliferative capacity. The clonogenic analysis demonstrated that inducible depletion of *Cdc42* from $Lgr5^+$ IESCs resulted in markedly reduced enteroid formation and Lgr5-GFP^+ IESC proliferation in a time-dependent manner (Fig. 7C). To confirm the genetic depletion data, we utilized CASIN, a selective CDC42 activity inhibitor,[45] to partially block GTPase function in the enteroids. Consistently, CASIN at 10 μM significantly reduced enteroid budding (Fig. 7D). *Cdc42* variants were identified as genetic risk factors of CRC, but functional genetic proof had so far not been performed.[14] In line with the important role of CDC42 as a key protein during colon crypt regeneration and in counteracting inflammation-induced colitis, we found that

depletion **(C)** Results are expressed as a graph of crypt buds per crypt or as the number of crypt buds versus time (days). One-way ANOVA was used to test for the variance of two groups (*$P < 0.01$). Representative enteroids over time are shown. **(D)** 2-[(2,3,4,9-Tetrahydro-6-phenyl-1*H*-carbazol-1-yl)amino]ethanol (CASIN), a selective CDC42 GTPase inhibitor, was used to partially block CDC42 signaling in the enteroids. CASIN at 5 or 10 μM significantly reduced enteroid budding. Representative enteroids are shown, from which budding curves were created. **(E)** Colonoids from colonic crypts were cultured and passaged into six parallel wells per mouse for each experiment ($n = 4$ mice per group). The colonoids were then exposed to 2 Gy irradiation in the absence or presence of 5 μM CASIN. Representative colonoids are shown. The number of crypt buds was counted daily for a minimum of 10 colonoids per well after CASIN-induced CDC42 inhibition. CASIN further reduced the number of crypt buds after irradiation. Scale bars = 200 μm. **(F)** Proposed model for CDC42 requirement in maintaining IESC and progenitor homeostasis (I); loss of CDC42 leads to susceptibility to GI diseases and aberrantly increased CDC42 promotes CRC progression (II).

Cdc42 depletion induced colon cancer phenotypes that were more vulnerable to irradiation injury (Fig. 5E). Thus, Lgr5^{+} intact colonic crypts were isolated and colonoids derived from colonic crypts were grown to determine whether pharmacological inhibition of CDC42 would sensitize colonic IESCs to irradiation. We subjected wild-type colonoids to 2 Gy γ-irradiation. We determined that after irradiation, all colonoids lost some proliferative capacity (Fig. 7E), but CASIN-treated colonoids showed significantly reduced budding compared to irradiation-treated control colonoids (Fig. 7E). Taken together, our *in vitro* experiments demonstrate that reduced CDC42 impairs crypt formation and sensitizes the colonic IECs to noxae that predispose to colitis and CRC development.

Discussion

The analysis of a cohort of colorectal cancer patients showed that high expression of CDC42 was a negative prognostic marker but was associated with good prognosis in chronic inflammatory diseases, such as IBD, arguing for a contradictory role of CDC42 in chronic inflammation in benign versus progressed colon neoplasia. In this article, we utilized unique mouse models of GI injury and state-of-the-art murine organoid cultures to show that CDC42 is essential for maintaining IESC→progenitor homeostasis and regenerative response to intestinal or colonic injuries (Fig. 7F—I), including colitis and tumorigenesis. Monogenic loss of CDC42 fails intestinal IESC regeneration and repair of the impaired IEC barriers that sequentially induce mucosal inflammation, which is known to play a causal role in intestinal crypt dysplasia in the context of aging that might result in CRC. We conclude that lack of intestinal CDC42 disrupts the homeostatic transition of IESCs to progenitors, leading to impaired IEC regeneration that induces GI mucosal inflammation, which is susceptible to colitis. In turn, the disrupted transition from IESCs to progenitors leads to aberrantly activated progenitor proliferation, crypt dysplasia and neoplasia along with increased CDC42 expression, which could promote colon cancer development or CAC progression (Fig. 7F—II).

CDC42 is classified into the Rho family of GTPases along with Rac 1 and RhoA, a family that is highly conserved in regulating epithelial morphogenesis, apicobasal polarity, and intercellular junction remodeling during development.[45—47] CDC42 signaling is switched "on" or "off" by its intrinsic GTPase activity between an inactive GDP-bound state and an active GTP-bound state to regulate various essential cellular processes during homeostases, such as cell cycling, migration, and fate determination.[48] Several studies reported that *Cdc42* mRNA expression or CDC42 kinase activity is increased in colorectal adenocarcinoma[13,49] and inhibition of CDC42 suppresses primary colon cancer growth in mice.[50] Conversely, deletion or reduction of CDC42 promotes epithelial hyperplasia and tumorigenesis in the liver and skin by disturbing β-catenin degradation.[51,52] These data suggest that epithelial CDC42 signaling has a dual role in the pro-oncogenic transformation and tumor suppression depending on downstream effectors and co-factors. Our murine data suggest that CDC42 is essential for protecting IESCs from intestinal injury and for preventing colonic IEC transformation into colorectal cancer. Because only scarce information exists on expression and activation levels of CDC42 in chronic inflammatory disease of the gut and associated colorectal cancer manifestation and progression, we undertook a detailed comparative pathology study. We found that CDC42 loss and low expression correlate with increased survival in colorectal cancer patients.

Aging or colitis can promote chronic intestinal inflammation to induce colorectal cancer, which involves multiple oncogenic pathways in IECs, such as *APC^{min}*, *KRAS*, and *p53*.[29,53—56] Sequential accumulation of activating and inactivating mutations in various oncogenes and tumor suppressors could drive the transition from normal IESCs at the crypt bottom to CSCs that lead to CRC.[19,53,57] Consistent with previous reports,[12] our results showed that depletion of *Cdc42* in IECs led to IESC progenitor hyperplasic proliferation and IEC dysplasia, and mucosal inflammation in the context of aging. In contrast, numbers of Lgr5^{+} IESCs as well as activity in response to irradiation injury were significantly reduced by either inducible or persistent depletion of IEC CDC42. Strikingly, we observed that depletion of *Cdc42* led to intestinal senescence-associated secretory phenotypes (Fig. S4C), and colonic neoplasia that developed as macroscopic adenomas located in the colorectal area after 9—13 months. These new findings suggest that a single mutation of *Cdc42* might induce colonic neoplasia when combined with other stress factors, such as aging and chronic injuries. However, the loss of *Cdc42* led to an increased crypt cell death, which most likely induced a compensatory proliferation from the TA population. Intriguingly, mice with loss of colonic *Cdc42* were more susceptible to colitic injury with decreased colonic Lgr5^{+} IESC regeneration and delayed mucosal healing. Furthermore, genetic or pharmacological inhibition of CDC42 impaired colonoid regeneration. Our data suggest that on the one hand, CDC42 is required for IESC homeostatic proliferation towards progenitors, and thus indispensable for colonic mucosal repair. On the other hand, loss of CDC42 may increase sensitivity to intestinal injury, which could be the cause of IBD susceptibility.

Interestingly, using *Olfm4Cre*ER^{T2}, a recent study showed that depletion of *Cdc42* leads to the disrupted apical-basal polarity in the IEC compartment and crypt hyperproliferation.[58] However, Olfm4 marks the small intestinal IESCs as well as their progenitors, and *Olfm4*-driven GFPCreER^{T2} expression induces the depletion of *Cdc42* in both small intestinal IESCs and progenitors in TA zone.[59] In contrast, Lgr5 marks the small intestinal and large crypt IESCs, and *Lgr5*-driven CreER^{T2} expression induces the depletion of *Cdc42* in the small and large intestinal IESCs.[60] Therefore, our data not only reflect the enteropathy in small intestines due to defective CDC42 but also provided evidence of CDC42 requirement to large bowels, where inflammation and neoplasia often occurred. Our future research might focus on whether CDC42 or *Cdc42* status in IBD patients or aged subjects is correlated with chronic mucosal inflammation and progression to CRC.

Wnt and Notch signaling pathways regulate IEC homeostasis and differentiation.[61] Constitutive activation of a Wnt target gene drives the formation of intestinal benign

adenomas[57,62]; by contrast, inhibition of Notch promotes IESC expansion towards a secretory fate to maintain the undifferentiated state in *Apc*-mutant neoplastic IECs.[63] Our data showed that depletion of *Cdc42* increased β-catenin nuclear abundance in the transformed colonic IECs, but the β-catenin expression did not display a significant alteration in the normal area of *Cdc42*-deficient colon or small intestines. Coincidently, the adenoma in the *Cdc42*-deficient mice lacked mucin, indicating that depletion of *Cdc42* led to the loss of *APC* and activation of β-catenin.[57] These data suggest that IEC CDC42 could be a neoplastic marker. In contrast, depletion of *Cdc42* leads to increased NICD1 expression in intestinal IECs and mislocalized Paneth cells, which is often senescence-associated intestinal inflammation (Fig. S6A). These data indicate an activated Notch signaling after depleting *Cdc42*, which might alter Paneth cell maturation. Although depletion of colonic *Cdc42* did not change NICD1 in the colon, *Cdc42*-depleted mice that were challenged by γ-secretase for 5 days, a Notch inhibitor, displayed ameliorated mucosal hyperplastic lesions in the small intestine (Fig. S6B). Nevertheless, these data suggest that loss of CDC42 function might differentially affect intestinal and colonic IESC/progenitors. In future research, we will use unbiased RNA sequencing to determine the transcriptional effects of CDC42 signaling upon both the Wnt and Notch pathways.

Clinical therapeutics targeting CSCs are being developed for CRC treatment.[64] CDC42 is newly identified as a cancer marker and is highly expressed in metastatic tumors.[13] Interestingly, our data showed that CASIN, a CDC42 GTPase inhibitor induced the loss of proliferation in the cultured colonoids post-irradiation. This finding was consistent with our murine data showing that intestinal and colonic IESCs lacking CDC42 are highly sensitive to irradiation injury. These data further suggest that high or low CDC42 expression could be relevant as a protein marker that indicates inflammatory response outcome upon irradiation treatment of CRC. Our studies are highly relevant to the pathogenesis of CRC in the setting of aging or colitis.

In summary, we utilized unique mouse models, human samples, and murine organoid culture with loss of CDC42 function to define a potential marker reflecting intestinal injury and CSC treatment outcomes. Therefore, our results present a novel therapeutic direction for CRC.

Conclusions

Depletion of *Cdc42* disrupts the IESC transition to active progenitors. Monogenic loss of *Cdc42* initiates intestinal neoplasia, leading to colorectal adenoma in the context of aging.

Author contributions

XH contributed to the study concept and design. XH, DZ, WT, HN, MS, TK, FK, MT, LD, and WT contributed to data acquisition. XH and YZ generated transgenic mice. XH, RM, YZ, HR, HD, TK, FK, JW, LK, and WT analyzed the data. YZ, RM, LK, FK, and HD contributed reagents/materials/analysis tools. XH wrote the manuscript.

Conflict of interests

All authors have no conflicting financial or non-financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.11.024.

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