CDC42 Inhibition Suppresses Progression of Incipient Intestinal Tumors

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Abstract

Mutations in the APC or β-catenin genes are well-established initiators of colorectal cancer, yet modifiers that facilitate the survival and progression of nascent tumor cells are not well defined. Using genetic and pharmacologic approaches in mouse colorectal cancer and human colorectal cancer xenograft models, we show that incipient intestinal tumor cells activate CDC42, an APC-interacting small GTPase, as a crucial step in malignant progression. In the mouse, Cdc42 ablation attenuated the tumorigenicity of mutant intestinal cells carrying single APC or β-catenin mutations. Similarly, human colorectal cancer with relatively higher levels of CDC42 activity was particularly sensitive to CDC42 blockade. Mechanistic studies suggested that Cdc42 may be activated at different levels, including at the level of transcriptional activation of the stem cell enriched Rho family exchange factor Arhgef4. Our results indicate that early-stage mutant intestinal epithelial cells must recruit the pleiotropic functions of Cdc42 for malignant progression, suggesting its relevance as a biomarker and therapeutic target for selective colorectal cancer intervention.

Introduction

Colorectal cancer remains the most prevalent digestive cancer, affecting nearly 150,000 people annually in the United States and mortality being an outcome for a third of these patients (1). The view that APC mutation acts as a genetic initiator for most colorectal cancers has been shaped by molecular pathologic observations and studies of colorectal cancer animal models (2, 3). APC mutations are detected in 88% of nonhypermutated colorectal cancers (4), and are found in the earliest microadenoma lesions containing only several dysplastic glands (5). Compared with other somatic mutations with progressively higher mutation frequency in advanced adenocarcinomas, APC mutations have a similar rate in both microadenomas and advanced adenocarcinomas (2, 6). The earliest morphologic changes in APC<sup>−/−</sup> mouse intestines (7) emerged as outpocketing epithelial pouches from the upper part of a crypt. These tumor-initiating cells then developed laterally into the neighboring villi forming the typical adenomatous polyps (7). Similar morphogenetic changes were described in mice carrying a dominant stable mutation in β-catenin (8), suggesting that some shared mechanisms might underlie the aberrant morphogenetic transformation in early-stage tumor cells.

APC directly binds to an APC-stimulated exchanging factor (Asef1, or Arhgef4 hereafter) through its conserved Armadillo repeat domain (9, 10). Both Arhgef4 and its homolog Spata13 (Asef2) are specific guanine nucleotide exchanging factors (GEF) for cell division control 42 (Cdc42; refs. 11, 12). Cdc42 is a small GTPase regulating various aspects of cell morphogenesis, division, and migration. APC directly interacts with Cdc42 in multiple in vitro interaction assays (13). The link between APC and Arhgef4 has been viewed as one of the most persuasive evidences for APC-mediated remodeling of cytoskeleton (14). However, with controversial mechanisms proposed for APC-mediated Cdc42 activation (12, 15, 16), the functional output of this regulatory cascade during intestinal tumorigenesis remains poorly understood.

Cdc42 was observed to be highly expressed in 60% of human colorectal cancers with its level positively correlating with poorly differentiated colorectal cancers (17). Arhgef4 was recently identified as one of the signature genes characteristic of Lgr5<sup>−/−</sup> intestinal stem cells (18). Both Arhgef4 and Cdc42...
have been proposed as tumor suppressors (12); however, in vivo evidence supporting this notion has been missing. We have recently showed that Cdc42 deletion impaired intestinal Lgr5\(^{+}\) stem cell homeostasis (19). Here, we provide genetic evidence that nascent mouse intestinal tumor cells carrying single mutations in either APC or \(\beta\)-catenin could activate Cdc42, possibly at different levels. Inhibition of Cdc42 by genetic ablation or small-molecule inhibitor attenuated tumorigenicity of the fast-cycling microadenoma-constructing tumor cells, whose survivability depended on high levels of cell-autonomous Cdc42 activity. Human colorectal cancers with higher Cdc42 levels are more sensitive to Cdc42 inhibition. Our results suggest that Cdc42 may be an immediate mediator of APC/\(\beta\)-catenin mutations in early-stage tumor cells, and may be used as a biomarker for selective targeting of some colorectal cancers.

### Materials and Methods

#### Mice

Cdc42\(^{fl/\text{loxP}}\) (20), Catnb\(^{\text{ex3)}}\) (8), Lgr5\(^{EGFP-IRES-creERT2}\) (21), Rosa26R\(^{CRE}\) (22), Villin-Cre (23), Villin-CreER (24), and Apc\(^{Min/+}\) mice (25) have been described previously. Mice were maintained at 129/BL6 mixed background. As reported previously (29), Proximal third of the small intestines were dissected and enteroids were cultured in glass bottom dishes (MatTek Corporation; catalog no. P35-1.5-10-C) and housed in plastic cages with temperature control 37-2 digital module and CTI Controller 3700 digital (Zeiss), respectively.

Isolation of Lgr5\(^{+}\) intestinal stem cells from 3-month-old Lgr5\(^{EGFP-IRES-creERT2}\) mice was performed with a four-way MoFlo cell sorter (Beckman-Coulter) based on EGFP and EpCAM expression as described previously (26).

#### Human colorectal cancer cell culture

Human colorectal cancer cells, LIM1863, LIM1899, LIM2551, LIM2550, LIM1215, SW480, and Caco2, have been maintained in conditions described previously (27, 28). For LIM1863 cells that form tumor organoids in suspension, cells were grown in RPMI-1640 (Corning; MT10-040-CM) with 10% FBS (Sigma; M3772) that form tumor organoids in suspension, cells were grown in conditions described previously (27, 28). For LIM1863 cells that form tumor organoids in suspension, cells were grown in RPMI-1640 (Corning; MT10-040-CM) with 10% FBS (Sigma; M3772) that form tumor organoids in suspension, cells were grown in conditions described previously (27, 28).

For passaging, LIM1863 cells were mechanically pipetted up-and-down to break the organoids before seeding into fresh medium. All in vitro cell experiments were carried out in triplicates and repeated at different circumstances.

To determine the growth rate of different cell lines, cells (10\(^7%/\text{well}\)) were seeded into a 96-well plate in triplicate. Cell growth was measured every 24 hours using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories). Briefly, WST-8 was reduced by dehydrogenases in cells to give an orange soluble formazan dye, which is directly proportional to the number of living cells. CCK-8 reagent (10 \(\mu\)L) was added into each well, incubated for 2 hours, and subjected to absorbance measurement at 450 nm. Cell culture media were gently replenished every 24 hours to avoid losing viable cells. For Cdc42 activity-specific inhibitor (CASIN) treatment experiments, 3 \(\times\) 10\(^5\) cells per well were seeded in 100-\(\mu\)L culture media in the absence or presence of 10 \(\mu\)mol/L CASIN. Cell growth was measured by CCK-8 every 24 hours. To avoid losing viable cells, suspended cells in culture media were also collected by centrifugation at 200 \(\times\) g and seeded back when culture media were replenished.

CASIN (C\(_{20}\)H\(_{22}\)N\(_2\)O\(_4\)), or 2-((6-phenyl-2,3,4,9-tetrahydro-1H-carbazol-1-yl) amino) ethanol, was dissolved in DMSO and administrated to culture media at desired concentrations (19). Cells were treated with same volumes of DMSO in control groups. Lentiviral particle-mediated Cdc42-knockdown (KD) has been described in detail previously (19).

For cell-cycle analysis by flow cytometry, cells were cultured until they reached a confluence of 70% to 80%, trypsinized, and harvested by centrifuge. After washing by PBS, 10\(^6\) cells were resuspended in 0.5-mL PBS, dispersed into single cells by pipetting. Cells were fixed by 70% ethanol at 4°C. Fixed cells were centrifuged, washed by PBS, incubated in 10 \(\mu\)g/mL propidium iodide (Sigma; P4170) with 0.1% (v/v) Triton X-100 and 100 \(\mu\)g/mL DNase-free RNaseA in PBS, at 37°C for 10 minutes, and subjected to flow cytometry analysis.

For live cell or cancer organoid imaging, small intestinal crypts or human colorectal cancer cells were cultured in glass bottom dishes (MatTek Corporation; catalog no. P35-1.5-10-C) and imaged using an inverted Zeiss Observer wide-field microscope equipped with a CoolSnapHQ\(^2\) (Photometrics). Axiovision 4.8 was used to acquire differential interference contrast images every 5 minutes using a 63 × water immersion objective for various durations depending on the experiments. The dish was maintained at 37°C in a 5% CO\(_2\) environment using temperature control 37-2 digital module and CTI Controller 3700 digital (Zeiss), respectively.

For colorectal cancer cell line xenograft assays, male athymic nude-Fox1 nu/nu mice (5-weeks old; Harlan Laboratories) were randomly allocated into two groups, 5 mice per group, and housed in plastic cages with filter tops and acclimated to the regular diet. Colorectal cancer cells grown in regular culture media without antibiotics were divided into two groups: one treated overnight (~16 hours) with 5 or 10 \(\mu\)mol/L CASIN and the other with DMSO. Cells (1 \(\times\) 10\(^6\)) were harvested in 100 \(\mu\)L of a 2:1 mixture of serum-free RPMI-1640 and Matrigel (BD Biosciences), and subcutaneously injected to the left and right flank of the mice (i.e., left for CASIN-treated cells, and right for control cells). For xenograft of colorectal cancer cells with stable Cdc42-KD, 2.5 \(\times\) 10\(^6\) cells of each group, that is, control and Cdc42-KD cells, were subcutaneously injected to the left and right flank of the nude mice (\(n = 5\) for each cell line). Tumor volume, weight, and food consumption were monitored once a week. Tumor size (length and width) was measured by a caliper and calculated on the basis of the formula (tumor volume = length \(\times\) width\(^2\) \(\times\) 0.5).

The tumors were dissected and weighed; one half of the tumor was fixed in 10% buffered formalin for histologic and IHC analyses and the other half was snap-frozen for biochemical analysis.

#### Mouse intestinal crypt isolation and enteroid culture

Procedures for crypt isolation and culture were essentially the same as reported previously (29). Proximal third of the intestines were dissected and flushed with 1 \(\times\) PBS, cut open...
and fragmented into smaller pieces (~2 cm sizes). Tissue fragments were then rinsed three times in PBS followed by two washes in cold chelating buffer (2 mMol/L EDTA in PBS) at 4°C for 5 and 40 minutes, respectively. Intestinal fragments were then vigorously resuspended in the cold chelation buffer and allowed to flow through 70-μm filter (BD Falcon, 352350) into precooled PBS. A pellet of crypts was obtained by centrifuging this flow-through suspension at 200 × g for 3 minutes at 4°C. The pellet was then washed twice and resuspended in cold PBS for counting. Of note, 100 to 200 crypts suspended in Matrigel (BD Biosciences, 356231) were plated into each well of precooled plate at 37°C 24-well plates. After allowing the Matrigel to solidify at 37°C for 10 minutes, 500 μL of ENR organoid culture medium was added to each well (30). Working ENR (EGF, Noggin, R-spondin) medium contains 2 × N2 supplement (Life Tech Gibco, 17502-048), 0.5 × B2 (Life Tech Gibco, 17504-044), 1 mmol/L N-acetyl cysteine (Sigma, A9165), 0.05 μg/mL of EGF (Life Technologies, PMG8043), 0.1 μg/mL of Noggin (Peprotech, 250-38), and 50 μg of R-Spondin (R&D Systems, 3474-RS-050) made in 50 mL of basal culture medium. A total of 200 organoids of each genotype were added to each well. Every following morning, surviving crypts in each well were counted under a fluorescent microscope from 4 to 6 wells for each genotype. For coculture experiments, wild-type and EGFP-knockout (Cdc42fl/fl-Cre;B6129S6G-EGFP) crypts, 100 total, were mixed at three different ratios: 25:75, 50:50, and 75:25, and surviving Cdc42-knockout (KO) crypts, identifi ed, counted, classifi ed, and measured. Neoplastic lesions were categorized into three subclasses: aberrant crypts (<0.25 mm), small adenomas (0.25–1 mm), and large adenomas (>1 mm).

For assessment of microadenomas in β-catenin–mutant mice, intestinal tissues were dissected out from neonatal mice of 5 to 12 days of ages, ﬁ xed in 4% paraformaldehyde, and embedded in parafﬁ n. H&E-stained tissue sections were analyzed by two experienced independent observers. The numbers of aberrant crypt-like foci (ACF) were counted for each villus. One hundred crypt-villus compartments were counted for each histology section for individual mice.

Quantitative real-time RT-PCR
TaqMan quantitative real-time RT-PCR (qRT-PCR) was carried out in triplicate for mouse Arhgef4, Cdc42, Spata13, and APC, using probe sets mm00805525_m1, mm01194005_g1, mm01323215_m1, and mm00545872_m1, respectively (Applied Biosystems). All assays were designed to have primers/probes to span exon–exon junctions. Relative gene expression was calculated by the ratio of the target genes to β-actin (mm00607939_s1) on the ABI StepOnePlus Detection System. Primers for Sox9, Lgr5, and CD44 are listed in the Supplementary Data. To examine mRNA expression from organoids, tamoxifen-treated wild-type and Ex3fl/fl–Vill-CreER organoids were used for RNA extraction. To examine the possibility of stem cell–specifi c transcriptional regulation of Arhgef4, we interrogated existing enhancer chromatin datasets (H3K4me2 ChIP-seq) for evidence of a stem cell–specifi c enhancer at the Arhgef4 locus |datasets in Gene Expression

Immunohistochemistry, confocal immunofluorescence, and RNA in situ hybridization
Procedures of immunohistochemistry and confocal immunofluorescent analyses have been described previously (31). Confocal fluorescent and differential interference contrast images were acquired using a Zeiss LSM 510 confocal microscope. Terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) staining (32) and RNA in situ (33) were performed as previously described. Primary antibodies were described in the Supplementary Data.

Western blot analysis
Western blot analyses for specifi c protein targets were performed on freshly prepared tissue or cell lysates following procedures previously described (19). To detect active Cdc42, GTP-bound Cdc42 was pulled down using GST-human PAK1-PBD supplied by Cdc42 activation kit (Cell Signaling Technology; catalog no. 8819), and detected by Cdc42 antibody (Cell Signaling Technology; catalog no. 2466). Experiments were repeated on independent biologic samples for multiple times.
**Cdc42 Favors Growth of Intestinal Tumor Cells**

Omnibus (GEO) GSM1246037 and GSM851222. Data were normalized for promoter chromatin levels and then visualized using the Integrative Genomics Viewer.

**Statistical analysis**

Oncomine analysis (34) was conducted in June 2014, using the research edition (www.oncomine.org), and queried the following datasets (35–41). Kaplan–Meier curves were generated for survival analyses. Morphometric analyses of intestinal villi and crypts were performed using the NIH ImageJ software. One-way analysis of variance (ANOVA) followed by Tukey post hoc test was used for the comparison of the differences between the control and experimental groups in nude mice tumorigenicity assays. Student t test was used to determine the difference between experimental and control groups. Statistical significance was indicated by P < 0.05 or P < 0.01 in two-tailed comparison. Mean values are shown in all graphs with error bars representing the standard error of the mean (SEM).

**Results**

**Cdc42 is essential for intestinal crypt maintenance**

Previous studies showed that total loss of Cdc42 in intestinal epithelial cells (IEC) impaired crypt organization in Cdc42<sup>−/−</sup>;Villin-Cre (or Cdc42<sup>fl/fl</sup>) mice (19), suggesting that crypt homeostasis depends on Cdc42. Western blot analyses using separated intestinal tissues showed the highest levels of Cdc42 and Cdc42-GTP (active form) in crypts, compared with villus and stromal compartments (Fig. 1A). Higher levels of Arhgef4, an APC-driven Cdc42-specific GEF, were also detected in crypts encompassing both the Paneth and Lgr5<sup>+</sup> stem cell domains (Fig. 1B). Adult Lgr5<sup>+</sup> stem cells (EGFP<sup>+</sup>) isolated by fluorescence-activated cell sorting (FACS) of Lgr5<sup>EGFP-ires-CreER</sup> mouse IECs showed significantly higher Arhgef4 levels than EGFP<sup>−</sup> IECs (Fig. 1C). This transcriptional enrichment of Arhgef4 in Lgr5<sup>+</sup> stem cells was supported by the presence of a stem cell–specific enhancer region upstream of Arhgef4 promoter (Fig. 1D, see arrows). Crypts isolated from Cdc42<sup>fl/fl</sup> mice (Fig. 1E) failed to establish intestinal organoids in culture, with 94% of these Cdc42-KO crypts lost within 24 hours (Fig. 1F) and less than 1% survived up to 72 hours (Fig. 1G), collectively suggesting that Cdc42 activity is essential for crypt survival and maintenance.

Paneth cells provide niche factors and support Lgr5<sup>+</sup> stem cell homeostasis (30), and were observed to be dislocated from Cdc42<sup>fl/fl</sup> mouse crypts (19, 42), raising the possibility that impaired Paneth cell development might indirectly contribute to observed loss of Cdc42-KO crypts (Fig. 1F). However, neither exogenous Wnt5a protein (Fig. 1G) nor coculture with wild-type crypts rescued these Cdc42-KO crypts (knockout crypts were labeled as EYFP<sup>+</sup> by a Rosa26<sup>EYFP</sup> allele; Fig. 1H). One out of 100 Cdc42<sup>fl/fl</sup> crypts (EYFP<sup>+</sup>) formed initial buds; however, none of these buds propagated as wild-type organoids for a prolonged duration of culture (not shown). These data suggested that Cdc42 deletion cell-autonomously affected stem cells.

**Reduction of Cdc42 alleviated APC<sup>Min/−</sup> mouse polyposis**

Cdc42 is overexpressed in the large majority of colorectal cancers (17). We used Oncomine analysis (34) and examined transcript changes in colon tumors compared with normal tissues from eight primary colon tumor datasets containing expression data for SPATA13, a homolog of ARHGEF4. Of these, seven showed a significant increase (P < 0.001; Student t test) in SPATA13 transcript levels in colon tumors (35–41). ARHGEF4 and CDC42 showed inconsistent patterns of expression. Although transcript expression levels may hint at a functional role in tumor development, the activation status of Cdc42 small GTPase is arguably more relevant. Using APC<sup>Min/−</sup> mice, we asked whether and when Cdc42 is activated during the intestinal tumorigenesis in this model. Western blot analyses detected increased Cdc42-GTP levels in APC<sup>Min/−</sup> mouse small and large intestines around 6 to 7 weeks (Fig. 2A). At this stage, dysplastic crypts became evident histopathologically (Fig. 2C), consistent with the reported earliest detection of intestinal lesions in this mouse model (43). These distorted glands frequently possessed eosinophilic granular debris (Fig. 2C, see high magnification), positively labeled by Paneth cell marker lysozyme (Supplementary Fig. S1A). Highly increased levels of both total Cdc42 and Cdc42-GTP levels were observed in older APC<sup>Min/−</sup> mice with IEC-specific deletion of one or both Cdc42 alleles. APC<sup>Min/−</sup> mice developed tumors preferentially in small intestines (25), matching the sites of Vil-Cre–mediated Cdc42 deletion. Western blot analyses showed the reductions of Cdc42 and Cdc42-GTP levels upon Cdc42 deletion from APC<sup>Min/−</sup> intestines (Fig. 2E). APC<sup>Min/−</sup>;Cdc42<sup>fl/fl</sup>;Vil-Cre mice with both Cdc42 alleles deleted, showed early postnatal growth retardation and diarrhea, phenotypes resulting from Cdc42 deficiency (19). Pronounced epithelial cystic formation and lack of Paneth cell were observed in the intestines of these mice; however, no polyps were detected (Fig. 2D and Supplementary Fig. S1B). We excluded APC<sup>Min/−</sup>;Cdc42<sup>fl/fl</sup>;Vil-Cre mice from long-term tumorigenesis assays due to phenotypic complexity and reduced body conditions, and performed the analyses on APC<sup>Min/−</sup>;Cdc42<sup>fl/fl</sup>;Vil-Cre and their littermate controls.

At 4 to 5 months of age, APC<sup>Min/−</sup> mice developed in average 50 polyps per mouse revealed by indigo carmine staining (Fig. 2F). In contrast, the number and size of polyps were significantly decreased in APC<sup>Min/−</sup>;Cdc42<sup>fl/fl</sup>;Vil-Cre mice throughout the entire small intestinal tract (Fig. 2F and G). This alleviation of polyposis was exhibited at molecular levels, as Cdc42 reduction also reduced the levels of Tcf1, Tcf4, Sox9, C-Myc—a key mediator of Wnt signaling–driven colorectal cancers (44) and nuclear β-catenin (Fig. 2E and F), suggesting that high levels of Cdc42 might be required for APC<sup>Min/−</sup> intestinal tumor progression.
Reduction of Cdc42 level attenuated intestinal tumorigenesis in β-catenin–mutant mice

Cdc42 stabilizes β-catenin in skin cells through a GSK3β-mediated mechanism (20). We reasoned that if Cdc42 deletion triggered β-catenin degradation, it would explain the observed tumor suppression in APCMin/+ mice. (Fig. 2G). On the other hand, this tumor-suppressive effect would not be observed in a β-catenin stable–mutant genetic background. To explore whether Cdc42 inhibition indeed attenuated intestinal tumorigenesis by changing β-catenin stability, we examined the colorectal cancer mouse model carrying a dominant stable mutation, Catnbfl(ex3), in β-catenin (8). Cre-mediated deletion of β-catenin exon 3 in this mouse allele generated a truncated protein missing the GSK3β phosphorylation sites, leading to a dominant stable hence "activated" β-catenin (8). Western blot analyses detected the truncated form of β-catenin in Catnb(ex3)/+;Vil-Cre mouse intestines carrying one allele of activated β-catenin (Fig. 3A). Remarkably, we detected a 14-
Figure 2. Cdc42 reduction alleviated APC<sup>Min/+</sup> intestinal polyposis. A, Western blot analyses showed increased levels of active Cdc42 in small and large intestines of 7-week APC<sup>Min/+</sup> mouse intestines compared with age-matched wild-type mice of same genetic background (left, compare lanes 1–3 with lanes 4–6). Total and active Cdc42 were increased in older APC<sup>Min/+</sup> mice (right). Col, colon; Ile, ileum; Jej, jejunum. B, Arhgef4 immunohistochemistry showed expansion of its expression domain into adenomatous tissues in APC<sup>Min/+</sup> intestines. C, dysplastic lesion in 40-day-old APC<sup>Min/+</sup> mice. Note that the distorted glands possess large eosinophilic granular debris, typically found in Paneth cells. D, total deletion of Cdc42 in Cdc42<sup>IEC</sup>; APC<sup>Min/+</sup> mice caused epithelial cystic formations. No dysplastic crypt was found. There is a complete loss of Paneth cell granule in crypts. E, Western blot analyses for total, active Cdc42, and several Wnt targets. Note that Cdc42<sup>fl/+</sup>;Vil-Cre littermate mice, phenotypically wild-type in terms of intestinal development and function (19), were used as reference. F, Cdc42 reduction decreased the tumor load in APC<sup>Min/+</sup> mouse intestines. Macroscopic polyp detection by indigo carmine staining was followed by histology analyses and β-catenin immunohistochemistry on swiss-roll intestinal sections to identify adenoma. Note that Cdc42 inhibition reduced β-catenin nuclear but not epithelial junction staining. All comparison was made from same intestinal segments from littermate animals unless specified otherwise. G, tumor numbers were counted and sizes were measured in duodenum, jejunum, and ileum of mice with indicated genotypes. ∗, P < 0.05 when compared with Cdc42<sup>fl/+</sup>;Vil-Cre; and †, P < 0.05 when compared with APC<sup>Min/+</sup> mice. Scale bars, 5 μm.
fold increase in Cdc42-GTP level in these mutant intestines (Fig. 3B). Total Cdc42 levels were increased about 3-fold in the same tissues (Fig. 3B), suggesting that the mutant cells might have activated Cdc42 from different levels, including transcriptional upregulation of Cdc42 itself. Approximately 50% of Catnb(ex3)/þ;Vil-Cre pups died within the first week of their life, while none survived up to 12 days (Fig. 3C, red line). Numerous ACF, or microadenomas (8), were found throughout intestinal villus epithelia (Fig. 3D, see arrows). These lesions contained proliferative cells identified by both bromodeoxyuridine (BrdUrd) and pH3 labeling (Fig. 3E and G).

We derived Catnb(ex3)/;Cdc42fl/fl;Villin-Cre mice, in which the Cdc42 gene dosage was reduced by half in the mutant IECs (Fig. 3B, gray bars). Western blot analyses confirmed a reduction of Cdc42-GTP levels in these double heterozygous mice (Fig. 3B). The medium survival time of these double heterozygous mice was significantly prolonged from 9 to 13 days, compared with Catnb(ex3)/;Villin-Cre
Cdc42 Favors Growth of Intestinal Tumor Cells

littermates (P < 0.001, Fig. 3C). Ten percent of animals were rescued up to 39 days of their life upon Cdc42 reduction (Fig. 3C, green line), accompanied by a significant gain of body weight (Supplementary Fig. S2). This improved survival was also reflected by the significant reduction of villus lesions (Fig. 3D and F) and reduced tumor cell proliferation (Fig. 3E–G). The residual proliferative epithelial lesions in double heterozygous animals (Fig. 3E and F) indicated that reduction of Cdc42 by half might not be sufficient to completely block tumorigenesis, because when Cdc42 was completely deleted in Catnb(ex3)/+;Villin-Cre mice, no ACF, but severe epithelial cystic formation, was observed (Supplementary Fig. S3). These data collectively suggested that genetic attenuation of Cdc42 also reduced intestinal tumorigenesis in a β-catenin stable–mutant background.

Cdc42 inhibition impaired survival of nascent microadenoma cells

The morphogenesis of crypt-like foci in early-stage APC–mutant (7) and β-catenin–mutant intestines (8) represented a typical pathologic feature of intestinal tumorigenesis. As Cdc42 regulates actin and microtubule organization, cell migration, and morphogenesis through an array of well-documented effectors (45), we asked whether Cdc42 might be directly involved in promoting such microadenoma morphology. Indeed, reduction of Cdc42 levels did significantly reduce crypt-like foci in the villi (Fig. 3D and F). Of note, tumor cells within these foci strongly expressed Paneth cell marker—lysozyme (Fig. 4A)—as well as Wnt-specific transporter (Gpr177) and Wnt ligand (Wnt3; Fig. 4A). Double staining demonstrated that tumor cells within these microadenoma foci were proliferative (Fig. 4A and B, see arrows pointing to BrdUrdþ nuclei). Upon genetic Cdc42 reduction, the number of these proliferative microadenomas was reduced (Fig. 4B and Supplementary Fig. S4), accompanied by a significant increase of cell death within the same microadenoma foci (Fig. 4D). Western blot analyses for cleaved caspase-3 further confirmed this increased apoptotic activity triggered by Cdc42 inhibition (Fig. 4C). Therefore, compared with cells outside of the foci, the fast-cycling tumor cells within these microadenoma foci appeared to be more sensitive or vulnerable to Cdc42 inhibition. Loss of these microadenoma cells was further supported by drastic reductions of Tcf4, CD44, and Sox9 levels following Cdc42 reduction (Fig. 4E).
Incipient intestinal tumor cells activated Cdc42 after acquiring mutation

To investigate whether Cdc42 activation is an early molecular event driving tumor progression from incipient intestinal tumor cells, we inducibly introduced dominant-active β-catenin mutations into Catnb(ex3)fl/fl;Villin-CreER intestinal organoids in culture via tamoxifen administration and examined Cdc42 activation statuses. Induced Catnb(ex3)fl/fl;Villin-CreER organoids developed cyst-like morphology, markedly different from the wild-type organoids (Fig. 5A). Real-time RT-PCR detected significantly increased levels of Arhgef4, along with expected Wnt target genes CD44 and Sox9 (Fig. 5C), suggesting that β-catenin mutation activated Arhgef4 at the transcriptional level. Despite a lack of transcriptional induction of Cdc42 expression (Fig. 5C), the total and activated Cdc42 protein levels were increased in Catnb(ex3)fl/fl;Villin-CreER organoids (Fig. 5D). Of note, the weak but clearly detectable Cdc42-GTP induction reflected a quantitative Cdc42 activation from 200 organoids; similar induction was not observed in Catnb(ex3)fl/fl;Villin-CreER organoids, suggesting that the Cdc42-GTP levels of the latter were below detection limit. Acute administration of C20H22N2O, a CASIN (19, 46, 47), abrogated the growth of both wild-type and Catnb(ex3)fl/fl;Villin-CreER tumor organoids (Fig. 5A), by inducing cell death revealed by propidium iodide staining (Fig. 5B), suggesting that these tumor organoids could not tolerate the total inhibition of Cdc42 activity.

Cdc42 inhibition reduced the tumorigenicity of Cdc42High human colorectal cancer cells

Intestinal tumor stem cells (TSC) might require higher Cdc42 activities to sustain their divisions. To determine whether the active Cdc42 level could serve as a useful indicator of effective tumor suppression by inhibiting this molecule, we examined a panel of well-characterized human colorectal cancer cell lines carrying distinct APC or/and β-catenin mutations (28). These cells were chosen because of their molecular, morphologic, and behavioral representations of clinical colorectal cancer features. Microsatellite stable (MSS) LIM1863 cells contain a truncated APC at amino acid 1,564 and wild-type β-catenin (28); LIM2550 cells, microsatellite instability (MSI) colorectal cancer, are hypermutated at APC, K-RAS, and PI3K loci; and LIM1215 cells (also MSI) are metastatic and carrying a deletion in β-catenin exon 3 (28). Western blot analyses showed that total and active Cdc42 levels were increased in organoids with induced β-catenin mutation. Data represent Cdc42 activities from seeded 200 organoids of each genotype.
Given that the fast-cycling Lgr5 stem cells expressed higher Arhgef4 than villus cells (Fig. 1C), we speculated that cell-cycle status might influence Cdc42 activities. We performed cell-cycle analysis using flow cytometry on individual colorectal cancer cell lines to examine whether a particular cell cycle phase would contribute to the observed differential Cdc42 activities. When we compared the cell cycle distributions among different cell lines, we found that the relative...
Cdc42 levels were not precisely correlated with their percentage of distribution at any certain cell-cycle phase (Supplementary Fig. S5A). Interestingly, LIM1863 showing the highest Cdc42 activity grew in liquid medium as floating organoids (Fig. 6B), previously described as crypt-like structures (48). Caco2 (19, 27) and LIM1899 cells were capable of forming three-dimensional epithelial cyst-like tumor organoids in Matrigel, whereas other cell lines tested under same conditions showed irregular or invasive growth behavior in three-dimension (data not shown).

Live cell imaging of tumor organoids showed that CASIN treatment abolished the proliferative activities normally observed at the peripheral of LIM1863 tumor organoids, causing a drastic accumulation of membrane protrusions at tissue surfaces (Supplementary Fig. S5B). The growth of all LIM1863 organoids was arrested within 48 hours by CASIN in culture (Supplementary Fig. S5B). Significantly reduced tumor formation was detected in nude mice injected with LIM1863 treated with 10 μmol/L CASIN (~16 hours; Fig. 6C–E). In contrast, LIM1215 and LIM2550 cells (with low Cdc42) exposed to 16-hour CASIN treatment formed tumors after subcutaneous injection (Fig. 6D). As LIM1899 and LIM2550 cells tolerated the short period of CASIN treatment (no significant growth arrest at 24 hours; Fig. 6F), but not a sustained CASIN treatment especially at their exponential growth phases (P < 0.01 at 48 hours; Fig. 6F), we speculated that these Cdc42-low tumor cells might be sensitive to a sustained Cdc42 deprivation. Thus, we performed stable Cdc42-KD in LIM1899, LIM2550, Caco2, and LIM1215 using Cdc42-specific lentiviral shRNA particles, and successfully established LIM1899, LIM2550, and Caco2 cell clones with stable Cdc42-KD (Fig. 6G); no stable clone was established for LIM1215 under same condition. Significant inhibition of tumor cell growth and tumorigenicity were observed in these Cdc42-KD colorectal cancer cells (Fig. 6H and I). These data suggested that Cdc42 inhibition was effective to a variety of colorectal cancers, in particular to those with high Cdc42 activities.

Discussion

More than 90% of colorectal cancers have APC and/or β-catenin mutations (4), and the majority overexpresses Cdc42 (17). The proposed molecular regulation of Cdc42 by full-length β-catenin (10) versus truncated APC (10) was controversial (9, 12), thus the role of this regulatory cascade during colorectal cancer pathogenesis was not clear. Both Cdc42 and Arhgef4 have been proposed to be tumor suppressors (12). Through genetic and pharmacologic analyses of mouse and human colorectal cancer models in vitro and in vivo, we have obtained several lines of evidence to support that some of colorectal cancer cells activate Cdc42 for immediate cell survival and microadenoma morphogenesis.

Aberrant morphogenesis of crypt-like foci represents one of the hallmarks of colorectal cancer pathogenesis (49). The molecular bases of such morphogenetic transformations, especially the very initial erratic movement of incipient tumor cells (7), have not been fully elucidated. We provided in vivo evidences that Cdc42 was strongly elevated and activated in both APC<sup>Min<sup>+/−</sup> and β-catenin-mutant mouse intestines. Of note, Cdc42 was immediately activated in IEC organoids following the inducible introduction of β-catenin mutations. In this APC<sup>wild-type</sup> genetic background, Cdc42 activation was likely activated by an increased amount of Arhgef4, whose transcriptional levels were significantly elevated upon β-catenin mutation acquisition. This transcriptional activation of Arhgef4 was consistent with recently reported enrichment of this factor in Lgr5<sup>+</sup> stem cell population, and was further affirmed by our detection of a stem cell–specific enhancer upstream of Arhgef4.

What advantages do tumor cells gain from Cdc42 activation? First, our results suggested that higher levels of Cdc42 activities might promote survivability of fast-cycling microadenoma-constructing tumor cells (Fig. 7). Intestinal crypts and stem cells haploid insufficient of Cdc42 (Cdc42<sup>−/−</sup>;Vil-Cre) behave normally, whereas intestinal tumor cells with β-catenin mutation underwent increased apoptosis following haploid insufficiency in Cdc42, suggesting that TSCs might demand higher Cdc42 for survival compared with wild-type stem cells. This notion was also supported by the facts that Cdc42 deletion attenuated APC<sup>Min<sup>+/−</sup> tumorigenesis and human colorectal cancers with higher Cdc42 activities were less tolerant to Cdc42 inhibitor. Prosurvival factors downstream of Cdc42, such as PAK1 (50), have been well documented, and our Cdc42 activation assays demonstrated consistently elevated Cdc42-PAK1 binding in both mouse and human colorectal cancers throughout the entire study. Cdc42 activation may also indirectly influence GSK3β activity through its direct target atypical PKC (20), ultimately promoting β-catenin-mediated cell survival and proliferation. A microRNA, miR185, that reduced Cdc42 expression also effectively induced cell-cycle arrest and apoptosis in cultured colorectal cancers (51). Thus, Cdc42 activation may cell-autonomously favor fast-cycling TSCs to survive and progress.

Second, we provided evidence that genetic inhibition of Cdc42 reduced the number of crypt-like foci and tumors in several mouse colorectal cancer models, whereas a Cdc42 inhibitor abrogated both mouse and human tumor organoids. Cdc42 activation in incipient tumor cells may immediately alter cell morphology through its well-established effectors such as IQGAP (52), reorganizing actin and microtubule cytoskeletons. This would directly favor tumor cell migration and aberrant tumor cell behaviors such as chromosomal...
misseggregation and aneuploidy, all of which could drive colorectal cancer progression.

Third, we showed that Cdc42 deletion also affected cells (e.g., Paneth cells) within the normal or TSC compartments. Total loss of Cdc42 perturbed the development of Paneth cells (19), the primary Wnt3 secretors in intestinal crypts. Loss of Wnt3 arrested the growth of intestinal organoids in vitro (53), a phenotype similar to Cdc42−/−MEC crypts. However, unlike Wnt3-deficient crypts, Cdc42−/−MEC crypts failed to be rescued by Wnt3a protein or coculture of wild-type crypts, indicating that Cdc42 was cell-autonomously required by stem cells for survival. The impact of Cdc42 on Paneth-like tumor cells was most likely indirect. On the other hand, reduction of Cdc42 clearly abrogated the formation of microadnoma foci that contained tumor cells with Wnt-producing capacity, suggesting that Cdc42 activation may also indirectly favor TSC growth through a non-cell-autonomous mechanism by assembling a tumorigenic microenvironment (Fig. 7).

A large fraction of APC mutations in colorectal cancers, including the Mlm mutation, result in premature stop codon and truncated proteins that retain the conserved N-terminus armadillo domain responsible for Arhgef4, Cdc42, and IQGAP binding (54). Structure and cell culture analyses led to conflicting interpretations of APC-mediated Cdc42 activation (9–12, 15, 16). Our data suggested that Cdc42 might be activated in colorectal cancers from multiple levels, including mechanisms depending on Arhgef4 and ones influencing the level of Cdc42 itself. Together with the observation that deletion of Arhgef4 reduced APCMin+/- mouse tumorigenesis (55), our data supported the tumor-promoting effect of this Arhgef4–Cdc42 cascade. Cdc42 inhibition suppressed the in vivo tumorigenicity of several colorectal cancer cell lines, especially those with higher Cdc42 levels such as LIM1863. The growth of LIM1863 cells as floating organoids and Caco2 cells as epithelial cysts probably reflected some benign features of these cell types. In contrast, cells with low Cdc42 activities, for example, the hypermutated LIM2550 cells, and metastatic LIM1215 cells were relative resistant to Cdc42 inhibition, and might represent advanced colorectal cancer types. Our xenograft assays suggested that the growth of even these cell lines was inhibited by stable Cdc42 depletion. The relatively high tolerance by these cells could attribute to their widespread genome anomalies. K-RAS activating mutation in LIM2550 cells may elicit a parallel survival pathway. Our mouse colorectal cancer studies suggested that Cdc42 activation might be an early molecular event, blockage of which could attenuate colorectal cancer progression. Determination of Cdc42 level of a given tumor cell type may identify its potential sensitivity to Cdc42 inhibition. Cdc42-inactivating mutations are rare in colorectal cancers (4), hinting its indispensable role in initial tumor cell survival. Cdc42 might be considered as a useful marker and a potential target for selective intervention of colorectal cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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