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Cdc42 Coordinates Proliferation, Polarity, Migration, and Differentiation of Small Intestinal Epithelial Cells in Mice

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Abstract

Background & Aims—Cdc42 is a Rho GTPase that regulates diverse cellular functions, including proliferation, differentiation, migration, and polarity. In the intestinal epithelium, a balance among these events maintains homeostasis. We used genetic techniques to investigate the role of Cdc42 in intestinal homeostasis and its mechanisms.

Methods—We disrupted *Cdc42* specifically in intestinal epithelial cells by creating *Cdc42^{flox}/flox-villin-Cre⁺* and *Cdc42^{flox}/flox-Rosa26-CreER⁺* mice. We collected intestinal and other tissues, and analyzed their cellular, molecular, morphologic, and physiologic features, compared with the respective heterozygous mice.

Results—In all mutant mice studied, the intestinal epithelium had gross hyperplasia, crypt enlargement, microvilli inclusion, and abnormal epithelial permeability. Cdc42 deficiency resulted in defective Paneth cell differentiation and localization without affecting the differentiation of other cell lineages. In mutant intestinal crypts, proliferating stem and progenitor cells increased, compared with control mice, resulting in increased crypt depth. Cdc42 deficiency increased migration of stem and progenitor cells along the villi, caused a mild defect in the apical junction orientation, and impaired intestinal epithelium polarity, which can contribute to the observed defective intestinal permeability. The intestinal epithelium of the *Cdc42^{flox}/floxvillin-Cre⁺* and *Cdc42^{flox}/flox-Rosa26-CreER⁺* mice appeared similar to that of patients with microvillus inclusion disease. In the digestive track, loss of Cdc42 also resulted in crypt hyperplasia in the colon, but not the stomach.

Conclusions—Cdc42 regulates proliferation, polarity, migration, and differentiation of intestinal epithelial cells in mice and maintains intestine epithelial barrier and homeostasis. Defects in Cdc42 signaling could be associated with microvillus inclusion disease.

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Keywords

Proliferation; Polarity; Conditional Deletion; MVID Model

The intestinal epithelium is one of the most vigorously self-renewing adult tissues.¹ The intestinal villi constitute the functional compartment containing a number of differentiated, post-mitotic cell types: absorptive enterocytes, mucus-secreting goblet cells, and hormone-secreting enteroendocrine cells. Paneth cells that secrete antibacterial peptides and growth factors, however, reside at the base of the highly proliferative crypts of Lieberkühn, which also contain the intestinal stem cell (ISC) and transient amplifying progenitor cells.² In the intestinal epithelium, the balance between multiple cellular activities, such as proliferation, differentiation, migration, and polarity is tightly regulated in order to maintain proper homeostasis.

As a member of the Rho GTPase family, Cdc42 regulates polarized cell growth.^{3–5} In epithelial cells, Cdc42 knockdown results in polarity defects and mislocalization of Par polarity proteins,^{6,7} and it has been proposed that the interaction between active Cdc42 and the Par6-Par3—atypical protein kinase C (aPKC) signaling complex establishes—epithelial cell polarity during directed cell migration and morphogenesis.^{8–10} Gene targeting studies have shown that either loss of Cdc42 (Cdc42^{-/-}) or gain of function (Cdc42GAP^{-/-}) in primary mouse embryonic fibroblasts can cause defects in adhesion, polarity, and migration, and Cdc42 is required for filopodia induction and cell cycle progression.^{11–16}

The establishment and maintenance of apical-basal polarity within a single cell and in the context of the proliferative tissue is a key feature of intestinal epithelium morphogenesis. Previous cell biology studies by in vitro culture suggest that Cdc42 is not critical for the establishment of apical-basal polarity or cell proliferation, but is involved in directing mitotic spindle orientation and polarized lumen formation of cell clusters.¹⁷ Three-dimensional organotypic culture studies of Madin-Darby canine kidney (MDCK) or Caco-2 cells have shown that the phosphatase PTEN and phosphatidylinositol (4,5)-biphosphate regulate Cdc42 and atypical PKC activities to generate the apical plasma membrane domain and that Cdc42 modulates tight junctions and membrane protein traffic to maintain apical-basolateral polarity.^{18,19} To this end, understanding how epithelial cell polarity, proliferation, and cell-lineage specification are coordinated in tissues/organs is an important question in development and adult tissue homeostasis, and the molecular mechanisms that integrate these cellular activities within a specific tissue architecture during epithelial morphogenesis have just begun to be elucidated. Recently, it was demonstrated that Cdc42 controls pancreatic cell fate and tubulogenesis.²⁰ In the nervous system, Cdc42 regulates neuronal polarity/axon outgrowth and migration,^{21,22} establishes apical-basal polarity, and maintains proper neural epithelial progenitor proliferation rates.^{23,24}

Given the appreciated tissue specific signaling role of Cdc42 in mammalian physiology,²⁵ it remains unanswered if and what role Cdc42 plays in maintaining the mammalian intestinal epithelium. Here we report that *Cdc42* loss in mouse intestinal epithelium significantly increases crypt expansion due to a general hyperplasia of the proliferating stem cells that is associated with a gross abnormality in junction and polarity machineries. We demonstrate that Cdc42 is required for Paneth cell differentiation and localization and for maintaining the polarized structure and permeability of the intestinal epithelium. Significantly, small intestinal loss of Cdc42 produces a microvillus inclusion disease (MVID)-like phenotype.

Materials and Methods

Animal Studies

The Cdc42 floxed mouse has been described elsewhere¹⁶ and Cdc42^{flx/flx} crossed with villin-cre mice provided by Sylvie Robine (Institut Curie, Paris, France) or Rosa26-CreER mice and controls were generated by standard pairings. All animal protocols were approved by the Cincinnati Children's Hospital Medical Center (CCHMC) Committee on the Ethics of Animal Experiments. All mice are housed in a specific pathogen free breeding barrier and cared for by trained technicians and veterinarians. Euthanasia was performed by CO₂ followed by cervical dislocation. To delete Cdc42 (Rosa26-CreER), adult mice were injected intraperitoneally with 1 mg of Tamoxifen dissolved in corn oil per day for 4 consecutive days and were sacrificed 5 days post induction. Bromodeoxyuridine (BrdU) was incorporated in intestinal tissue by intraperitoneal injection of 2 mg BrdU (Sigma Aldrich, St Louis, MO) in phosphate-buffered saline (PBS). Tissues were collected at 2, 4, 6, 12, 24, and 36 hours post injection.

Intestinal weight measurements—Small intestines were collected from 2 month-old mice, PBS perfused, weighed, and normalized to total body weight.

Histology and Analysis

Intestinal tissues were flushed with PBS and fixed in 10% formalin or 4% paraformaldehyde overnight at 4°C. Tissues were then embedded in paraffin or cold optimum cutting temperature embedding medium. Processing of tissues for paraffin-embedding and H&E staining was performed by the CCHMC Digestive Health Center Morphology Core. Alcian blue was used to detect goblet cells and nuclei were counterstained with nuclear fast red.

For transmission electron microscopy, small intestinal sections were dissected, flushed with cold PBS supplemented with protease inhibitors and fixed in 4% glutaraldehyde/0.175 M cacodylate buffer followed by 1% osmium tetroxide/0.175 M cacodylate.

Antibody and Alkaline Phosphatase Staining

Antibodies used for Western blotting or immunofluorescence imaging are described in the Supplementary Materials. Alkaline phosphatase staining was performed on fresh frozen sections using a Vector Red Alkaline Phosphatase Substrate kit (Vector Laboratories, Burlingame, CA).

Microscopy

Mouse tissues for transmission electron microscopy were dehydrated, embedded in LX-112 resin, and imaged by the CCHMC Digestive Health Center Morphology Core. For human intestine samples, the small bowel biopsies were fixed in 3% glutaraldehyde embedded in LX-112 plastic and were evaluated on a Hitachi 7600 transmission electron microscope after uranyl lead acetate staining (Leica Microsystems, Buffalo, Grove, IL). Cofocal microscopy measurements are described in the Supplementary Methods.

Intestinal Epithelium Isolation and Western Blotting

Intestinal epithelium consisting of villi and crypts were isolated according to Guo et al.²⁶ Tissues were then homogenized in lysis buffer containing protease and phosphatase inhibitors, sonicated at 4°C, mixed with 4× sodium dodecyl sulfate loading buffer and heated at 100°C for 5 minutes. A Bradford assay was used to determine protein concentration (Bio-Rad, Hercules, CA). The following antibodies were used for Western blotting: Cdc42, RhoA, RhoC, and p-Histone-3 (Cell Signaling Technology, Danvers, MA);

Rac-1 and Par-3 (Millipore, Billerica, MA); PAR 6A, and anti-cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA); β -catenin, (BD Biosciences, San Jose, CA); LC3B Lamin B (Abcam, Cambridge, MA); glyceraldehyde-3-phosphate dehydrogenase (Fitzgerald Industries International, Acton, MA).

Crypt cells isolated as described here were used to separate cytosolic and nuclear fractions using the kit FOCUS SubCell (G Biosciences, St. Louis, MO).

Human Studies

For human samples, a de-identified case of MVID and a small bowel biopsy from a patient illustrating normal mucosa were obtained through the Cincinnati Biobank Tissue Repository in the Division of Pathology at CCHMC (Institutional Review Board protocol # 2010-0964).

Statistics

Results are expressed as mean $\pm$ SD. Analyses were performed using analysis of variance. $P < .05$ was considered significant.

Results

A Mouse Gene Targeting Model of Cdc42 Deficiency in Intestinal Epithelium

To examine the function of Cdc42 in the mammalian intestinal epithelium, intestinal epithelium-specific villin (vil)-cre expressing mice^{26–28} were crossed with Cdc42^{flox/flox} mice¹⁶ to generate Cdc42^{flox/flox}-vil-Cre⁺ (hereafter called Cdc42 knockout [KO]) mice, that lack the wild-type *Cdc42* gene in the small intestine and colon at 3 weeks of age (Figure 1A; Supplementary Figure 1A and B) and Cdc42 protein is also absent in both the crypt and villus intestinal compartments (Supplementary Figure 2). Compensatory expression of Rho GTPase family members was not detected (Figure 1A). Cdc42 heterozygous (Cdc42^{Wt/flox}-vil-Cre⁺) and Cdc42 KO mice were born in normal Mendelian ratios (Supplementary Table 1), but Cdc42 KO mice were significantly smaller in size and weighed less than Cdc42 heterozygous littermate controls (Figure 1B and Supplementary Figure 3). Cdc42 KO mice exhibit rapid deterioration with ruffled coats, hunched postures (data not shown), and distended abdomens consistent with dehydration by 3 months of age (Supplementary Figure 4). Cdc42 KO animals have a dramatically reduced lifespan of only 2–7 months (Figure 1C). No intestinal bleeding or diarrhea was observed in Cdc42 KO mice, but stomach swelling and constipation were noted (data not shown). To rule out Cdc42 deletion in off-target tissues, such as the heart, liver, and kidney as contributing to the observed Cdc42 KO phenotypes, we performed a Western blot and H&E staining of organ tissues and detected no Cdc42 protein reduction or histological abnormalities (Supplementary Figure 5). Cdc42 KO mice also have normal blood cell counts (Supplementary Table 2). Overall intestinal morphology is shorter, thicker, and more dilated in 1- to 6-month-old Cdc42 KO mice (Figure 1D) and the intestine/body weight ratio is increased 3-fold (Figure 1E), suggesting intestinal hyperplasia. Functionally, the Cdc42 KO intestine fails to maintain a proper barrier as evidenced by defective paracellular permeability and bacterial translocation to the draining lymph node (Figure 1F). Cdc42 plays an important regulatory role in maintaining gross intestinal morphology and permeability.

Cdc42 Deletion Causes Intestine Hyperplasia and Increased Crypt and Villus Dimensions

Two- to 6-month-old Cdc42 KO mice exhibit general intestinal and colon hyperplasia. The intervillus space is dramatically reduced due to epithelial hypertrophy and resulting compression of the lamina propria (Figure 2Ai–vi). Interestingly, Cdc42 KO crypt dimensions were increased significantly with a concomitant increase in cell number and

volume (Figure 2C). Overall villus architecture is affected: the Cdc42 KO villi are significantly longer (Figure 2B) and H&E staining (Figure 2A), as well as subsequent immunofluorescence labeling (see Figures 3 and 4) reveals a disruption of the highly organized epithelial nuclei alignment. These results suggest that Cdc42 deficiency causes gross morphologic and growth abnormalities in the intestine epithelium.

Differentiation of Paneth Cells Is Defective in Cdc42-Deficient Intestinal Epithelium

The characteristic cytosolic granules typical of Paneth cells, as visualized by H&E staining, were mostly absent in Cdc42 KO crypts (Figure 2Aiii-iv). To examine if Paneth cell localization or differentiation is affected by Cdc42 KO, lysozyme immunostaining was performed. As shown in Figure 3A and B and Supplementary Figure 6, normally crypt base-localized, lysozyme-positive cells were displaced to the villi and were fewer in number in 1- to 2-month-old Cdc42 KO mouse small intestinal tissue. In agreement, Paneth cell markers lysozyme and matrix metalloproteinase 7 messenger RNA expression is significantly reduced compared with controls (Figure 3C; Supplementary Figure 7). In addition, the Cdc42 KO lysozyme-positive cells were double-positive for lysozyme and the normally goblet cell-specific Alcian blue (Figure 3D), suggesting that the displaced Cdc42 KO lysozyme-positive cells likely represent a goblet/Paneth intermediate progenitor population. Next we examined the expression of several other intestinal epithelial cell lineage-specific markers. In contrast to Paneth cells, goblet and enteroendocrine cell number was unaffected, as detected by Mucin-2 and chromogranin A staining, respectively (Figure 3E and F). Likewise, enterocyte number detected by Glut5 staining was similar between control and Cdc42 KO (data not shown). Cdc42 specifically regulates proper Paneth cell differentiation and localization.

Increased Cell Proliferation and Hyperplasia in Cdc42 KO Intestinal Crypts

The majority of crypt cells, with the exception of Paneth cells, are actively cycling. Interestingly, the proliferating crypt cell population, marked by Ki67 (all non-G₀ cycling cells) and BrdU (S-phase), is significantly expanded in Cdc42 KO intestinal tissue, resulting in a deeper crypt (Figure 4A) that was consistent in all small intestinal regions (Figure 4B). Consistent with the hyperproliferative crypt cell phenotype, Cdc42 KO crypt cell homogenates showed significantly higher expression of several cell cycle markers, including, p-Histone-3 and cyclin D1, compared with controls (Figure 4C). Because Paneth cells were recently demonstrated to contribute to the local ISC microenvironment, or niche, the Cdc42 KO phenotypes of Paneth cell displacement and crypt hyperproliferation could suggest altered ISC properties. In support, messenger RNA levels of the ISC/early progenitor marker, *Olfm4*, are elevated in Cdc42 KO intestinal tissue as demonstrated by in situ hybridization (Figure 4D). CD133, another putative ISC/early progenitor marker, is also increased in Cdc42 KO crypts (Supplementary Figure 8). A terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling and cleaved caspase-3 staining showed that the Cdc42 KO intestinal epithelium did not undergo increased levels of apoptosis compared with controls (Supplementary Figure 9A and B). Additionally, autophagy activity was unchanged as detected by LCIIIIB Western blotting (Supplementary Figure 9C). It is therefore possible that Cdc42 is required for restricting the number and/or proliferative capacity of an ISC population, which might be associated with its essential role in regulating Paneth cell differentiation and localization.

Interestingly, the hyperplastic phenotype of the Cdc42 KO intestine is not associated with an induction of an inflammatory environment, as determined by real-time polymerase chain reaction of proinflammatory tumor necrosis factor— α , interleukin-6, and interleukin-10 cytokines, F4/80 staining for macrophages, and myeloperoxidase staining for neutrophils, confirmed that Cdc42 loss does not elicit intestinal inflammation (Supplementary Figure

10). In addition, the villin-Cre mouse model also deletes *Cdc42* in the colon, which also appears hyperplastic and disoriented in epithelium as revealed by H&E and Ki67 staining (Supplementary Figure 11).

Cdc42 Deficiency Leads to Impaired Epithelial Polarity, Altered Localization of Basolateral Proteins, and an Increased Migration Rate

To further investigate the abnormalities in *Cdc42* KO mice, the pattern of expression of markers of tight junction, adherens junction, polarity, and basolateral and basement compartments, were examined. Real-time polymerase chain reaction and Western blotting experiments showed that the messenger RNA or protein levels of several junction and polarity molecules, ie, E-cadherin, β -catenin, aPKC, and ZO-1, were not significantly affected in *Cdc42* KO intestinal tissues (Figure 5A and B; Supplementary Figure 12). β -catenin protein in total cell lysate, cytosol or nuclear fractions, as well as p- β -catenin, was mostly unchanged (Figure 5B). Interestingly, although loss of *Cdc42* in the intestines did not affect E-cadherin expression, it caused a disorganized junction orientation (Figure 5C) that was associated with reduced Par3 and Par6 expressions (Figure 5A, Supplementary Figure 12A). Consistently, the expression of Na^+/K^+ ATPase, a typical basolateral marker, was significantly reduced and more diffusely localized in the *Cdc42* KO (Figure 5D). However, laminin, a basement marker, was similarly distributed in *Cdc42* KO mice and controls (Figure 5D), and enterocyte microvilli and goblet cell thecae appear to be appropriately apically localized, indicating that the overall epithelial apico-basal polarity is intact. These results suggest that *Cdc42* deficiency causes a mild defect in the apical junction orientation and impairs intestinal epithelium polarity, which can contribute to the observed defective intestinal permeability.

By a BrdU chase method, in which mice were examined at 4, 6, 12, 24, or 36 hours post—BrdU pulse labeling, we observed a significantly faster crypt to villus migration rate of BrdU retaining cells in the *Cdc42* KO mice compared with controls (Figure 5E). These results indicate that *Cdc42* is a negative regulator of intestinal epithelial cell migration.

Cdc42 Deficiency Results in a MVID-Like Phenotype

Transmission electron microscopy of *Cdc42* KO villi showed the presence of abundant enterocytes with misaligned nuclei and shortened microvilli (Figure 6A). Interestingly, a significant portion of these *Cdc42* KO enterocytes contained a large intracellular lumen covered with microvilli-like structures that were alkaline phosphatase—positive demonstrating an ectopic expression of villi (Figure 6A; Supplementary Figure 13). It was also evident from the transmission electron microscopy analysis that the vacuoles that accumulated in *Cdc42* KO epithelial cells were mostly absent in the controls (Figure 6A). This phenotype is reminiscent of human MVID, a severe intestinal disorder characterized by an accumulation of microvesicles and microvilli-containing inclusion bodies in the intestine epithelium, as demonstrated by a MVID patient sample analyzed in parallel to *Cdc42* KO by transmission electron microscopy and anti-CD10 staining (Figure 6B).²⁹

Inducible Deletion of Cdc42 Results in Similar Intestinal Defects as Constitutive Cdc42 Deletion

To rule out the possibility that the observed *Cdc42* KO intestinal phenotypes are due to developmental defects caused by *Cdc42* deletion during embryogenesis and neonatal development, we used a Tamoxifen-inducible *Cdc42*^{flax/flax}-Rosa26-Cre-ER mouse model that effectively deletes *Cdc42* within days in adult mice. As shown in Figure 7A, at 5 days post induction, mutant mice exhibited the same abnormal intestinal morphology, including increased crypt depth and width as *Cdc42*^{flax/flax}-vil-Cre⁺ mice. Immunostaining for lysozyme revealed a clear mislocalization and reduction of the lysozyme-positive cells in

agreement with the constitutive Cdc42 KO model (Figure 7B). Interestingly, no gross abnormality was observed in the stomach tissue, where Cdc42 was effectively deleted in this mouse model (Supplementary Figure 14). The small intestinal crypt cells also displayed significantly increased proliferative markers, Ki67 p-Histone-3 (M-phase marker) cells (Figure 7C; Supplementary Figure 15). Finally, E-cadherin and PKC λ immunostaining showed a more disorganized and slightly diffused pattern in the inducible Cdc42 KO villi and crypts under a confocal microscope by quantification (Figure 7D, Supplementary Figure 16). These results strongly suggest that the phenotypes and molecular changes observed in the Cdc42^{flox/flox}-vil-Cre⁺ mouse model are not due to developmental defects.

To further examine the cellular role of Cdc42 in primary murine small intestinal crypt cells, we used an in vitro enteroid culture system to study the requirement of Cdc42 for crypt epithelial cell expansion and differentiation. As shown in Supplementary Figure 17, Cdc42-inducible deletion resulted in reduced enteroid budding and a mislocalization of Paneth cells without significantly affecting the epithelial cell AJ structure revealed by E-cadherin staining. The later lack of effect on AJ junction is similar to what reported in Caco-2 or MDCK cell lines after Cdc42 knockdown by short-hairpin RNA,^{17,18} suggesting that the abnormal intestinal phenotypes of Cdc42 KO are likely to be related to intestinal epithelial cell maintenance and differentiation, not a direct impact on intestinal epithelial cell junction sites.

Discussion

Recent mouse gene knockout studies in diverse tissue/organ cell types have provided powerful genetic evidence for physiological roles of Cdc42 in mammals that, in some circumstances, contradicts conventional wisdom of previous in vitro cell biology studies.^{25,30} It appears that Cdc42 plays tissue cell type- and pathway-specific roles in different physiological settings. Using a conditional gene targeting approach, we show here that small intestinal Cdc42 is essential for maintaining mouse epithelial homeostasis and permeability. In the absence of Cdc42, a general intestine hyperplasia develops along with severe alterations in mucosal permeability, resulting in death. Significant crypt enlargement with drastically increased cycling stem/progenitor cells is evident. These results demonstrate that Cdc42 negatively regulates cell proliferation in intestinal epithelium. Although a multitude of studies have suggested a role for Cdc42 in positively controlling cell proliferation, primarily in in vitro cell culture studies, several recent mouse genetic studies in hematopoietic stem/progenitors, neural progenitors, and other cell types have yielded results implicating Cdc42 as also negatively regulating cell growth in defined physiological contexts. One study of hepatocyte-specific Cdc42 deletion reported chronic liver damage, hepatomegaly, and development of hepatocellular carcinoma³¹ consistent with the notion that Cdc42 can act as a liver tumor suppressor. The hyperproliferative phenotype resulting from intestinal Cdc42 deletion suggests a similar role for Cdc42 in controlling intestinal epithelial cell growth, and loss of Cdc42 can serve as one hit leading to hyperplasia.

The mechanism by which *Cdc42* loss results in cell hyperproliferation remains unclear, and whether a Cdc42 pathway directly intersects with a cell cycle suppressive, rather than a cell cycle promoting, signaling pathway, or if an indirect event of the Cdc42-controlled signaling is involved, needs to be defined. Interestingly, the hyperproliferative phenotype was also observed in the adherens junction components α -catenin³² or p120-catenin^{33,34} gene targeted mice. Like Cdc42, p120-catenin is essential for the maintenance of barrier function and intestinal homeostasis in mice.³⁴ Intestinal E-cadherin deficiency caused disrupted epithelial lining and defective Paneth cell maturation and positioning.³⁵ Indeed, tissue polarity maintenance could be one way in which Cdc42 contributes to cell growth control. Most internal epithelial tissues contain a monolayer of polarized epithelial cells enclosing a

central lumen. In 3D culture of MDCK cells, Cdc42 establishes polarized epithelial cysts by regulating the vesicular trafficking of apical proteins. This Cdc42-mediated apical exocytosis occurred in a Par6-aPKC—dependent manner and is required for lumen formation.³⁶ In addition to its ability to regulate epithelial polarity via modulation of protein trafficking to distinct cellular surfaces, the Cdc42-Par6-aPKC signaling complex has been shown to control the formation of normal 3D polarized epithelial cyst structures by altering mitotic spindle orientation and the direction of cell division. In particular, suppression of Cdc42, or Par6/aPKC, resulted in aberrant cysts with multiple lumens in vitro.^{17,18,37} Here, ablation of Cdc42 caused the formation of intracellular lumen, not unlike that described by Kesavan et al.,²⁰ during pancreas development in a Cdc42-deficient mice model, in which deletion of Cdc42 in the developing pancreas caused a loss of apical polarization and microlumen formation. Cdc42 has also been shown to regulate neural progenitor fate and proliferative status,²³ and the effects are associated with mislocalization or loss of expression of the polarity components E-cadherin, Par6, aPKC, F-actin, and/or Numb.²⁴ In skin cells, Cdc42 controls progenitor cell differentiation, β -catenin turnover and cell-to-cell contact.³⁸ Cdc42 activity is required for sustaining E-cadherin—mediated adhesion at the adherens junction and linking adherens junctions to the actin cytoskeleton.^{39,40} The exact mechanism of Cdc42-regulated gene expression or stability of the adherent or polarity machine remains unclear, but it is known that Cdc42 can mediate gene expression through multiple pathways.^{12,25} It is possible that defective regulation of epithelium polarity and/or the dynamics of adherens junction on *Cdc42* deletion serves as an early event that in turn affects the proliferative capability, migration rate, and permeability of intestine epithelial cells.

Another consequence of intestinal *Cdc42* deletion was that the differentiation of crypt-based Paneth cells, but not other villus cell types, were severely inhibited. Cdc42 deficiency produced a lysozyme/mucin-2 double-positive cell population, possibly intermediate cells, located along villi, while the crypt-localized Paneth cells were undetectable. Paneth cells are comparatively long-lived, with a lifespan of 4 to 6 weeks in mice. Our finding of mislocalization and abnormal maturation after acute *Cdc42* deletion using a Tamoxifen-inducible model further shows that Cdc42 is continuously required for the maintenance of Paneth localization and maturity. Because Paneth cells have been recently recognized as niche cells that support a proliferative ISC population in the crypt,⁴¹ one possibility is that Cdc42 deficiency deregulates ISC in the crypts after niche cell loss.

We observed in the *Cdc42* KO mice a significant phenotype of microvillus inclusion and microvesicle accumulation in the intestinal epithelium, mimicking that of MVID. MVID is a severe congenital intestinal disorder and one of the leading causes of infant malnutrition. The standard diagnostics for MVID is small intestinal biopsy followed by electromicroscopy detection of the characteristic inclusion bodies lined by microvilli.²⁹ Although understanding of the mechanism of this disease remains rudimentary, a recent human genetic study has revealed that nonsense or missense mutations of *MYO5B*, a gene encoding for a myosin motor protein, is associated with MVID.⁴² Our Cdc42 KO mice represent another MVID-like model confirmed at the electromicroscopy level in addition to a Rab8a knockout mouse model,⁴³ which might be connected with MYO5B in a signaling cascade. A recent report linking Cdc42 and Rab8a signaling in regulating intestine epithelial polarity is consistent with our findings here,⁴⁴ suggesting that Cdc42, as a master regulator of actomyosin machinery, can engage intracellular secretory pathways to sustain the proper direction and dynamics of trafficking of basolateral membrane components.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

aPKC	atypical protein kinase C
BrdU	bromodeoxyuridine
CCHMC	Cincinnati Children's Hospital Medical Center
ISC	intestinal stem cell
KO	knockout
MDCK	Madin-Darby canine kidney
MVID	microvillus inclusion disease
PBS	phosphate-buffered saline
vil	villin

Figure 1. Global abnormalities caused by intestinal epithelium-specific *Cdc42* deletion in mice. (A) Western blot analysis of *Cdc42* and related Rho GTPase family members (RhoC, RhoA, Rac1) from duodunal, jejunal, and ileal small intestinal tissue in *Cdc42* heterozygous and *Cdc42* KO mice. (B) Male *Cdc42* KO and *Cdc42* heterozygous control mice at 1 month of age. (C) Kaplan-Meier survival curve of *Cdc42* KO compared with control littermates. (D) Comparison of gastrointestinal gross morphology in control and *Cdc42* KO mice (arrowheads). (E) Small intestine/body weight ratios of *Cdc42* KO and control littermates. $n = 25$ mice each genotype. $*P < .01$. (F) Small intestinal paracellular permeability to fluorescein isothiocyanate-dextran was determined using the everted gut sac method in control and *Cdc42* KO mice (upper panel). Bacterial translocation to draining mesenteric lymph nodes was determined as described in Methods (lower panel). $n = 9$ mice per genotype. $*P < .01$.

Figure 2.

Phenotypic effects of *Cdc42* deletion in intestine epithelium. (A) H&E staining of longitudinal and transverse duodenum intestinal sections from control (*i*, *iii*, *v*) and *Cdc42* KO (*ii*, *iv*, *vi*) 2-month-old mice. Scale bars = 100 μm. (B) Average duodenal villus height in 3-month-old *Cdc42* KO and control mice. *n* = 40 villi per mouse, 4 mice total. **P* < .01. (C) Quantifications of the average duodenal crypt width and depth from 3-month-old *Cdc42* KO mice and control littermates. *n* = 45 crypts per mouse, from 10 mice. Error bars = standard deviation. **P* < .01.

Figure 3.

Cdc42 deficiency causes a Paneth cell differentiation defect. (A) Paneth cells are mislocalized in duodenum of Cdc42 KO 2-month-old mice. Lysozyme (Paneth cells), green. 4',6-diamidino-2-phenylindole (DNA), blue. Scale bars = 100 μ m. (B) Quantification of lysozyme-positive cells in duodenal villi and crypts of 2-month-old mice. Error bars = standard deviation. n = 5 mice each genotype. $**P < .01$. (C) Messenger RNA expression of Paneth cell markers was examined in isolated duodenal small intestinal crypts from control and KO mice. Results are normalized to glyceraldehyde-3-phosphate dehydrogenase expression and expressed as fold change. $*P < .01$. (D) Deletion of Cdc42 affects differentiation of Paneth cells. Alcian blue (goblet cells), blue; lysozyme (Paneth cells), brown; nuclear fast red (DNA), red. Left panel arrows, Paneth cells at crypt base in controls; right panel arrows: double-positive Alcian blue and lysozyme-positive cells in Cdc42 KO. Scale bars = 100 μ m. (E) Goblet and enter-oendocrine cell numbers are unchanged between control and Cdc42 KO. Left panels, chromogranin A (enteroendocrine cells) green; right panels, Mucin2 (goblet cells) green. 4',6-Diamidino-2-phenylindole (DNA), blue. Scale bars = 50 μ m. (F) Quantification of goblet and neuroendocrine cells per villus per small intestinal region in control and Cdc42 KO. Error bars = standard deviation. n > 5 per genotype.

Figure 4.

Cdc42 deficiency causes small intestinal epithelial hyperproliferation. (A) Expansion of proliferating cells in duodenal crypts from 3-month-old control and *Cdc42* KO mice. Ki67 (actively cycling cells), red. BrdU (2 hours post injection, S-phase), green. Scale bar = 50 μ m. (B) Average number of BrdU-positive cells per crypt in each small intestinal region. Results are expressed as the percentage of BrdU-positive cells per total crypt cells. $n > 40$ crypts. Error bars = standard deviation. $n = 5$ per genotype. $*P < .01$. (C) Western blotting for p-Histone H3 and Cyclin-D1 in control and *Cdc42* KO duodenal crypt samples. (D) In situ of Olfm4 messenger RNA in *Cdc42* KO and control duodenum. Scale bar = 50 μ m.

Figure 5.

Cdc42 deficiency results in defective polarity and junction protein expression and altered basolateral protein distribution. (A) Relative gene expression of cell junction and polarity molecules in Cdc42 KO mice. Messenger RNAs isolated from the intestine of the respective animals were subject to quantitative real-time polymerase chain reaction analysis, and the results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. $n = 3$ mice per genotype $*P < .01$. (B) β -catenin protein was assessed in total, cytosolic, and nuclear fractions from Cdc42 KO and control duodenal crypts. GAPDH and Lamin B were used as markers for the cytosolic and nuclear compartments, respectively. (C) Expression and distribution of E-cadherin, green, in Cdc42 KO and control mice. Magnification: 20 $\times$. (D) Laminin, green (top panels) and Na/K ATPase, green (lower panels) in the duodenum. Magnification: 20 $\times$. (E) Relative control and Cdc42 KO duodenal crypt cell migration rates. Left panel, BrdU (S-phase), green. Right panel, quantification of BrdU-positive cell migration over time, standardized to the position of BrdU-positive crypt cells at 2 hours post injection. $n = 3$ mice each genotype. Scale bar = 50 μ m. $*P < .01$.

Figure 6.

Cdc42 knockout results in a MVID-like phenotype. (A) Electron micrographs of control (i) and *Cdc42* KO (iii) intestinal epithelium are shown at 12,000 $\times$ magnification. Pictures correspond to the mid-villus region. Arrows, microvillus inclusions in *Cdc42* KO animals. (ii) and (iv) alkaline phosphatase (AP) staining of control and *Cdc42* KO intestines. Scale bars = 50 μ m. (B) Human patient MVID phenotypes. (i) microvillus brush border from a normal small bowel mucosa. The enterocytes are covered by a uniform layer of microvilli. The cytoplasm shows normal subcellular organelles without any inclusions. (iii) microvillus brush border from a patient with microvillus inclusion disease. The surface of the enterocyte shows an interrupted layer of poorly formed microvilli and the cytoplasm contains inclusions lined by microvillous structures. (ii) CD10 immunostaining of the normal brush border, and (iv) shows CD10 staining of the sample from a patient with coarse irregular staining in the enterocyte layer. Magnification: 40 $\times$.

Figure 7.

Inducible deletion of *Cdc42* leads to similar defects in intestinal epithelial proliferation, Paneth cell differentiation, and E-cadherin distribution. (A) H&E staining of small intestinal duodenum of Tamoxifen-induced *Cdc42*^{flox/flox}-Rosa26-Cre-ER mouse. Scale bar = 100 μm. Immunofluorescent staining of (B) lysozyme (red), (C) Ki67 (red), and (D) E-cadherin (red) and PKCλ (green), and (B, C, D) 4',6-diamidino-2-phenylindole (blue), were examined in control and *Cdc42*^{flox/flox} Rosa26-Cre-ER⁺ intestinal epitheliums. (B), (C) Scale bars = 100 μm. (D), A indicates apical surface. Scale bars = 10 μm.