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3 **Proper E-cadherin membrane location in colon requires Dab2 and it**
4 **modifies by inflammation and cancer**
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9 **Short running title: E-cadherin membrane location requires Dab2**
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52 human samples.
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ABSTRACT

We reported that Disabled-2 (Dab2) is located at the apical membrane in suckling rat intestine. Here, we discovered that, in colon of suckling and adult mouse and of adult human, Dab2 is only at lateral crypt cell membrane and colocalized with E-cadherin. Dab2 depletion in Caco-2 cells led to E-cadherin internalization indicating that its membrane location requires Dab2. In mice we found that 3 days of dextran sulfate sodium-induced colitis increased Dab2/E-cadherin colocalization, which was decreased as colitis progressed to 6 and 9 days. In agreement, Dab2/E-cadherin colocalization increased in human mild and severe ulcerative colitis and in polyps, being reduced in colon adenocarcinomas, which even showed epithelial Dab2 absence and E-cadherin delocalization. Epithelial Dab2 decrement preceded that of E-cadherin. We suggest that: Dab2, by inhibiting E-cadherin internalization, stabilizes adherens junctions and its absence from the epithelium may contribute to development of colon inflammation and cancer.

KEYWORDS: Dab2, E-cadherin, junctions, colon, inflammation, cancer

1. INTRODUCTION

The intestinal epithelium acts as selectively permeable barrier allowing the absorption of nutrients and simultaneously preventing the entry of luminal pathogens into the organism. The apical junction complex (AJC), composed of tight (TJ) and adherens (AJ) junctions, connect adjacent cells (see Pawłowska & Prof. Sobieszcańska, 2017 for a review). AJ consist of the transmembrane proteins E-cadherin and nectins and of cytosolic proteins, such as p-120, β -catenin and α -catenin, which mediate E-cadherin anchorage to the actomyosin cytoskeleton. The TJ transmembrane proteins include claudins, occludin, JAM and marvelD3 and among the cytosolic proteins is zonula occludens-1 (ZO-1) that interacts with transmembrane proteins and with the actin cytoskeleton, giving stability to the junction. The formation of TJ is preceded by and depends on AJ formation (see Miyoshi & Takai, 2005 for a review). The AJC are dynamic structures where new proteins are supplied to and recycled from the junctions and its maintenance is crucial for both, keeping the epithelium polarity and the intestinal barrier integrity. The alteration of the intestinal barrier integrity leads to the development of intestinal and extraintestinal diseases (see Fukui, 2016; Lechuga & Ivanov, 2017 for review).

Disabled-2 (Dab2) is a cytoplasmic adaptor protein (Xu, Yang, Jackowski, & Rock, 1995), that by interacting simultaneously with cytosolic and membrane proteins, plays multiple physiological roles through endocytosis, such as polarized cellular trafficking of membrane receptors, adhesion molecules as E-cadherin, signalling molecules, the cystic fibrosis transmembrane conductance regulator in intestine, among others (see Finkielstein & Capelluto, 2016 for a review); it participates in Gap junctions endocytosis (Piehl et al., 2006) and in immune responses modulation (see Finkielstein & Capelluto, 2016 for a review), and it is required for epithelial cells surface positioning in the endoderm (Rula et al., 2007) and for endosome recycling (Penheiter et al., 2010). Dab2 has also been considered a tumour suppressor because its expression is reduced or lost in several human cancer types (see Finkielstein & Capelluto, 2016 for a review), including colon cancer (Kleeff et al., 2002). This reduction appears to contribute to epithelial-mesenchymal transition (EMT) in breast, cervical, ovarian and pancreatic cancer cells (Chao et al., 2012; Hocevar, 2019; Martin, Herbert, & Hocevar, 2010; Piao et al., 2017). The evidence regarding the role of Dab2 in inflammation is contradictory and focused on immune cells (see Finkielstein & Capelluto, 2016 for a review).

We reported that the Dab2 located at the apical membrane of the suckling rat small intestine epithelium might mediate intestinal endocytosis of milk macromolecules

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3 (Vázquez-Carretero, García-Miranda, Calonge, Peral, & Ilundáin, 2011). In the current
4 study, we discovered that Dab2 is involved in E-cadherin localization at cell-cell
5 junctions in Caco-2 cells and that Dab2 and E-cadherin colocalization is disrupted in
6 colon inflammation and adenocarcinoma in mouse and human *ex vivo*.
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2. MATERIALS AND METHODS

2.1 Materials

The antibodies and their dilutions used in the current work were anti-Dab2 (Santa Cruz) 1:100; anti-Dab2 (Proteintech) 1:50; anti-occludin (Invitrogen) 1:400; anti-ZO-1 (Invitrogen) 1:200; anti-E-cadherin 1:300; anti- β -catenin (Sigma-Aldrich) 1:500 for immunofluorescence and anti-Dab2 (BD Transduction Laboratories) 1:1,000; anti-occludin (Invitrogen) 1:2,000; anti-ZO-1 (Invitrogen) 1:1,000; anti-E-cadherin (BD Transduction Laboratories) 1:1,000; anti- β -catenin (Sigma-Aldrich) 1:2,000; anti- α -tubulin (homemade) 1:10, for Western-blot. Unless otherwise indicated the other reagents used in this study were from Sigma-Aldrich (Spain).

2.2 Cell culture

Stable human epithelial colorectal adenocarcinoma cells (Caco-2) were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 20% of fetal calf serum and in the presence of 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C and in a 5% CO₂ atmosphere. Cells were cultured and plated for experiments as previously described (Elbediwy et al., 2012).

2.3 Depletion of Dab2 in Caco-2 cells

RNA-mediated interference (RNAi) experiments were used. Caco-2 cells were cultured to 80% confluence and transfected with either a pool of siRNAs specific for Dab2 (siDab2) or a non-targeting control siRNAs. In both cases, the total final siRNA concentration was 0.2 μ M. For the transfection, Interferin transfection reagent (Polyplus Transfection) was used according to the manufacturer's instructions. The siRNAs were purchased by Sigma-Aldrich. The sequences of the specific siRNAs for Dab2 were (5'...3'): CAAUCGACACCUUCUUCGU and ACGAAGAAGGUGUCGAUUG; CAUGAUGACUUUGAUGCUA and UAGCAUCAAGUCAUCAUG. 3 days after transfection, samples were collected and processed either for immunofluorescence, western blot or paracellular gate permeability.

2.4 Analysis of the paracellular gate

Ion permeability was determined by measuring transepithelial electrical resistance (TER). A silver/silver-chloride electrode was used to determine the voltage deflection induced by an alternating current square wave current (± 20 μ A at 12.5 Hz) using an epithelial voltohmmeter (EVOM; World Precision Instruments) as previously described (Matter & Balda, 2003). Briefly, either control or siDab2 Caco-2 cells were plated on transwell filters and allowed to form confluent monolayer in normal medium. TER was

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3 measured after 24, 30, 48 and 60 h of culture and results are expressed in $\text{ohm} \times \text{cm}^2$.
4 Paracellular permeability of hydrophilic tracers was monitored by measuring the
5 transmonolayer flux of either 4 kDa FITC- or 70 kDa Rhodamine B-conjugated dextran
6 over 2, 4, 6 and 8 h. Fluorescence was then determined with a FLUOstar OPTIMA
7 microplate reader (BMGLabTech, Offenburg, Germany).
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11 **2.5 Animals and experimental colitis**

12 C57BL/6 mice of 8 day (suckling) and 3 month-old (adult) were housed in a 12:12
13 light/dark cycle and fed *ad libitum* with normal rodent diet (Harlan Ibérica S.L.) and free
14 access to tap water. They were humanely handled and sacrificed by cervical
15 dislocation according with the guidelines of the European Union Council (Directive
16 2010/63/UE).
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21 Colitis was induced in 3 month-old mice by drinking water containing 3% (wt/vol)
22 dextran sulphate sodium (DSS, MW 40 kDa; TdB Consultancy) during 3, 6 or 9 days.
23 Tap water was administered to DSS-untreated animals. Following sacrifice, the colon
24 was removed, washed with ice-cold saline solution and fixed by overnight incubation
25 with phosphate buffer saline (PBS) (in mM, 137 NaCl, 2.7 KCl, 10 Na_2HPO_4 and 1.8
26 KH_2PO_4 pH 7.4) containing 4% para-formaldehyde for histological analysis. The
27 progression of the colon inflammation was assessed by determining disease activity
28 index (DAI), histological score and by the mRNA levels of the pro-inflammatory
29 cytokines IL-1 β and TNF α , as described (Carvajal et al., 2017). For DAI evaluation,
30 daily, throughout the DSS-treatment, animals were monitored for weight loss, stool
31 consistency and blood in the faeces (scale of 0-3). Histological score (0–3 scale) was
32 based on destruction of epithelium, dilatation of crypts, loss of goblet cells,
33 inflammatory cell infiltrate and oedema (Supplementary Figure 1).
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43 **2.6 Human tissue samples**

44 Embedded-paraffin sections of: i) colon of patients with mild and severe ulcerative
45 colitis, ii) non-adenomatous colon polyps, iii) colon adenocarcinoma and iv) healthy
46 colon located away from the adenocarcinoma region, were obtained from 25 patients (5
47 patients per each condition), between 49- and 74-year-old, who had undergone colon
48 resection, through the "Biobanco del Sistema Sanitario Público de Andalucía, Hospital
49 Universitario Virgen del Rocío, Sevilla, Spain". Tissues were subjected to a
50 pathological examination at the Hospital to confirm the diagnosis of the pathology. The
51 study was approved by the Ethic Committees of Sevilla University and of Virgen del
52 Rocío Hospital. Informed consent was obtained from all patients.
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2.7 Immunofluorescence assays

Immunofluorescence was performed on: i) ileum and colon from healthy untreated mice, ii) colon from untreated and DSS-treated mice, iii) healthy colon, ulcerative colitis, non-adenomatous polyps and adenocarcinoma samples from patients, and iv) transfected Caco-2 cells. Transfected Caco-2 cells were grown on glass coverslips for 3 days and fixed directly in methanol at -20°C for 10 min. $5\ \mu\text{m}$ -paraffin embedded intestinal slides were boiled with 10 mM sodium citrate, pH 6, during 10 min. After blocking the intestinal sections for 1h (3% bovine serum albumin, BSA; 3% fetal calf serum, and 0.1% triton x100 in PBS) and the Caco-2 cells for 10 min (0.5% BSA and 20 mM glycine in PBS), they were incubated either without (controls) or with the primary antibody at 4°C , overnight. In the intestinal samples the primary antibody binding was visualized with the appropriate secondary antibodies: either Alexa Fluor -488 (1:100, Invitrogen) or -546 (1:100, Life technologies), and in Caco-2 cells with either FITC or Cy3 (1:500, Jackson ImmunoResearch Inc.). Nuclei were stained with Hoechst 33258 (Invitrogen). The intestinal slides were mounted (Vectashield, Vector) and photographed with an Olympus BX61 microscope equipped with a DP73 camera. Coverslips containing the Caco-2 cells were mounted (ProLong Gold, Invitrogen) and visualized with a DMIRB Leica microscope with a camera (C4742-95, Hamamatsu Photonics). Images were analysed using NIH ImageJ program. Quantification of colocalization was done by 3 independent observers by counting the total number of cells in 12 crypts well oriented longitudinally per sample and number of cells with yellow-orange colour signal per crypt. The results are expressed as the number of cells (either total or with yellow-orange colour signal) per crypt. The immunofluorescence intensity signal of Dab2 and E-cadherin was measured in 12 crypts well oriented longitudinally per sample and the results are expressed in arbitrary units. Negative controls without primary antibody were run in parallel (Supplementary Figure 2)

2.8 Western blot assays

Caco-2 cell were collected by washing twice with PBS and after SDS-PAGE sample buffer addition, the cells were homogenized with a 23G needle and heated at 70°C for 10 minutes. Samples were then processed using standard western blotting techniques. Protein-bound antibodies were detected with horseradish peroxidase conjugated secondary antibodies using enhanced chemiluminescence detection system (GE Healthcare Select®). Anti- α -tubulin antibody was used to normalize band density values.

2.9 Statistical analysis

Data are presented as mean \pm SEM. The number of animals or patients used for each condition was 5. One-way ANOVA followed by Tukey's test was used for multiple comparisons (GraphPad Prism Program v8.0). For comparisons between two groups, Student's *t* test was used. Differences were set to be significant for $p < 0.05$.

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3. RESULTS

3.1 Localization of Dab2 in the small and large intestine of suckling and adult mice

In the current work we investigated Dab2 function in adult mouse and human intestine under physiological and physiopathological conditions. We started the work determining by immunofluorescence the location of Dab2 protein in colon of 8 day- and 3 month-old mice and comparing the observations with those obtained in the ileum. As previously, in the suckling ileum the anti-Dab2 antibody specific signal was strong at the apical membrane of the cells lining the villus (Figure 1). In the adult ileum, Dab2 signal was seen at both apical and lateral cell membranes of the villus and crypt cells. The absence of Dab2 signal at the lateral cell membrane of suckling intestine might be due to the strong apical signal that precludes emerging the much less strong signals from the lateral membrane.

In the suckling and adult colon, Dab2 signal is mainly at the lateral membranes of the crypt cells, being the signal intensity stronger at the bottom of the crypts. The colonocytes apical membrane was slightly stained mainly in the suckling mice. The results suggest that the main function of Dab2 in adult intestinal epithelial is related to lateral membrane processes.

3.2 Dab2 depletion disrupts epithelial cell-cell apical junctions

As Dab2 was detected at the epithelial lateral membrane we tested whether it regulates the AJC by silencing its gene expression (siDab2) in Caco-2 cells (siRNA technique) and analysing the expression (western blot) and location (immunofluorescence) of TJ (ZO-1 and occludin) and AJ (E-cadherin and β -catenin) proteins. The western blot revealed that the silencing procedure reduced the expression of the two Dab2 isoforms by about 70% (Figure 2a). Tight junction appearance, as detected by occludin and ZO-1 staining, changed from a linear organization into a wavy structure (Figure 2b). In Dab2-containing Caco-2 cells, E-cadherin mainly appeared at the cell membrane as a linear structure and as a few E-cadherin spots in the cytoplasm. These spots likely might represent E-cadherin localization in cytosolic vesicles. Dab2 depletion affected E-cadherin membrane localization and increased its staining in the cytosol. This E-cadherin redistribution was accompanied by increases in cytosolic β -catenin (Figure 2b). Western blots show that Dab2 depletion did not significantly changed the abundance of the AJC proteins assayed (Figure 2c). These observations suggest that Dab2 is required for proper AJC organization, mainly AJs.

Since the observed siDab2-induced alterations in ZO-1 and occludin distribution might modify the barrier properties of the TJ, we next measured the TER and the

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3 paracellular dextran permeability in either control or siDab2 Caco-2 cells monolayers.
4 Neither the TER nor the dextran permeability (Supplementary Figure 3) were affected
5 by siDab2, indicating that the AJC disruption induced by 70% Dab2 depletion is not big
6 enough as to significantly modify the paracellular permeability.
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9 10 **3.3 Colocalization of Dab2 and E-cadherin in the small and large intestine** 11 **of suckling and adult mice**

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13 The most prominent effect of siDab2 on Caco-2 cells AJC was the change in E-
14 cadherin distribution; we next determined Dab2 and E-cadherin colocalization in mouse
15 intestinal epithelium by immunofluorescence. In all the samples examined, the anti-E-
16 cadherin antibody signal was seen at the lateral cell membranes (Figure 3). Dab2
17 colocalized with E-cadherin at the cell lateral membrane in both, ileum and colon. In the
18 suckling ileum colocalization was observed at the crypt-villus junction and along the
19 crypt-villus axis in the adult. In both, suckling and adult colon, colocalization was mainly
20 detected at the bottom of the crypts (Figure 3). These observations suggest that, as in
21 Caco-2 cells, in mouse intestinal epithelium the E-cadherin membrane location is
22 related to Dab2.
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29 30 **3.4 DSS-treatment effects on the expression and localization of Dab2 and** 31 **E-cadherin in mice colon**

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33 Since the available evidence indicates that mucosal inflammation induces E-cadherin
34 downregulation or mislocalization, we evaluated, by immunofluorescence, whether
35 inflammatory conditions (DSS-treatment during 3, 6 and 9 days) affected the colon
36 expression and localization of Dab2 and E-cadherin. Supplementary Figure 1
37 summarized the indicators of the colon inflammation progression. Inflammation was
38 relevant by day 3 of DSS-treatment, though some of the inflammatory parameters were
39 mildly increased. Compared with untreated colon, 6- and 9- days treatment increased
40 the levels of the pro-inflammatory cytokines by about 100 and 1000 times, respectively.
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46 As compared with DSS-untreated mice (see Figure 4), 3 days of DSS-treatment
47 increased: i) the number of cells expressing Dab2 along the entire crypt (Figure 4a); ii)
48 the intensity of the Dab-2 and E-cadherin signals at the lateral cell membranes, cytosol
49 and apical membrane domain (Figure 4a and b), and iii) the number of crypt cells and
50 colonocytes showing Dab2 and E-cadherin colocalization even at the apical membrane
51 (Figure a and c). Colocalization extends to about two-thirds of each crypt, instead of
52 only at the bottom. After 6 days of DSS-treatment the normal structure of the colon
53 epithelium was destroyed (Figure 4a) and therefore epithelial Dab2 and E-cadherin
54 signal intensity was decreased (Figure 4b). The remaining epithelium exhibited less
55 Dab2 at the lateral membranes (Figure 4a) and, consequently, decreased Dab2/E-
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3 cadherin colocalization (Figure 4c). This colocalization was absent after 9 days of DSS-
4 treatment (Figure 4a). These observations might indicate that the intestine firstly
5 responds to tissue injury by increasing epithelial Dab2 and E-cadherin to strengthen
6 the AJC and hence provide protection against inflammation. After 9 days, the
7 inflammatory inducers lead to depletion of epithelial Dab2 and E-cadherin.
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10 11 **3.5 Dab2 and E-cadherin localization in human healthy and pathological** 12 **colon** 13

14 We next determine whether the Dab2 and E-cadherin response to experimental colitis
15 found in mice also occurs in human colon. Immunofluorescence was performed on
16 colon specimens from patients with mild and severe ulcerative colitis, with non-
17 adenomatous colon polyps and colon adenocarcinoma and from healthy colon. As in
18 mouse colon, in human healthy colon Dab2 and E-cadherin are located at cell lateral
19 membrane and both proteins colocalized mainly at the bottom of the crypts (Figure 5).
20 In mild ulcerative colitis specimens, the E-cadherin signal was found at the lateral cell
21 membrane, at the apical membrane domain in some cells and in the cytosol; Dab2
22 signal was mainly at lateral membranes (Figure 5). The intensity of both, Dab2 and E-
23 cadherin signals were greater than in healthy colon (Figure 7a) and the number of crypt
24 cells showing Dab2 and E-cadherin colocalization was increased (Figure 7b). In severe
25 ulcerative colitis, expression and colocalization of Dab2 and E-cadherin is still higher
26 than in healthy colon but only seen in those cells that still maintained the epithelial
27 integrity (Fig 5, 7b and 7c).
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30 Since ulcerative colitis is associated with increased risk of colon cancer
31 development and Dab2 is considered to act as a tumour suppressor, we examined
32 Dab2 localization in human colon polyps and adenocarcinomas. Polyp specimens
33 (Figure 6) showed strong Dab2, E-cadherin and Dab2/E-cadherin signals at the crypt
34 cells lateral membranes and cytosol, higher than that in healthy colon and similar to
35 that in colitis (Figure 7). In the adenocarcinoma regions (Figure 6) where the epithelium
36 was partially preserved (adenocarcinoma 1), Dab2 and E-cadherin appeared as diffuse
37 membranous and cytosolic signals surrounding the nucleus, being the E-cadherin
38 signal at the membrane more abundant than that of Dab2. Some colocalization of Dab2
39 and E-cadherin was also observed. In other regions (adenocarcinoma 2), Dab2 was
40 completely absent from the remaining epithelium-like structures whereas E-cadherin
41 was still present. According to these observations, the disappearance of Dab2 from the
42 intestinal epithelium precedes that of E-cadherin and these findings, together with
43 those in a mouse model of experimental colitis and Dab2-depleted Caco-2 cells, might
44 suggest that the loss of Dab2 may lead to epithelial E-cadherin delocalization.
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3 In 9 days DSS-treated mice colon, in human severe ulcerative colitis and colon
4 adenocarcinoma, Dab2 is abundantly expressed by non-epithelial cells appearing in
5 the mucosa and submucosa. Some of them are macrophages and lymphocytes as they
6 present Dab2/arginase-1 colocalization (Supplementary Figure 4).
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10 **4 DISCUSSION**

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13 In the suckling rat ileum Dab2 localizes at the apical membrane domain and may
14 participate in the endocytosis of milk components (Vázquez-Carretero, García-Miranda,
15 Calonge, Peral, & Ilundáin, 2011). Herein we show that with age Dab2 localization
16 shifts from the apical to the lateral epithelial cells membrane of mouse intestine,
17 wherein it appears to contribute to AJC stability, which, in turn, might provide protection
18 against intestinal pathology development. Thus, Dab2 is required for the proper AJs
19 organization in Caco-2 cells, it co-localizes with E-cadherin at the cell-cell junctions in
20 mice and human intestinal epithelium and its expression and colocalization with E-
21 cadherin in colon is modified by pathological conditions.
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27 TJs and AJs are dynamic structures that are remodelled in a variety of
28 physiological and pathological situations, and their stability is critical to allow
29 physiological compartmentalization and targeted during disease. Our data are the first
30 demonstration of Dab2 localization at the AJC *ex vivo* and that Dab2 depletion induces
31 E-cadherin internalization in Caco-2 cells. Consequently, E-cadherin delocalization was
32 accompanied by β -catenin internalization. Dab2 depletion mildly affected TJs because,
33 though it induces a loss of tension of the ZO-1 and occludin, TJ permeability was not
34 affected. The small response of TJs to Dab2 depletion might indicate that either Dab2
35 is not important for TJ or that 70% Dab2 depletion was no enough to modify the TJs.
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42 Inflammation increases AJC proteins endocytosis and decreases their exocytosis
43 to the lateral cell membrane but mechanisms poorly understood (see Lechuga &
44 Ivanov, 2017 for review). Our results agree with those showing mislocalization of E-
45 cadherin during intestinal inflammation (see Lechuga & Ivanov, 2017 for review) and
46 suggest that Dab2 is involved in this mislocalization. At the early stages of inflammation
47 (3 days DSS-treatment), the colon responds to DSS-injury by increasing the intensity of
48 Dab2 and E-cadherin signals and the number of epithelial cells co-expressing the two
49 proteins at the lateral membrane. Coexpression of the two proteins is also observed at
50 the apical membrane domain of epithelial cells. Yang, Cai, Roland, Smith, & Xu, (2007)
51 proposed that directional transport and removal of apical E-cadherin by Dab2-mediated
52 endocytosis is important for bilateral targeting. According to this proposal, our results
53 suggest that at the beginning tissue injury increases the synthesis of Dab2 and E-
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3 cadherin that were initially exported to the apical membrane, from where E-cadherin
4 would be removed and targeted to the lateral membrane by Dab2- mediated
5 endo/exocytosis. The increase in epithelial Dab2 and E-cadherin could be an initial
6 tissue response to strengthen the AJs and hence, attempt to provide protection against
7 colon inflammation. Accordingly, an inflammation-protecting role could be attributed to
8 epithelial Dab2. Progression of mouse colon inflammation, from 3 to 6 and 9 days-
9 treatment, is accompanied by epithelial Dab2 and E-cadherin downregulation,
10 decreased Dab2/E-cadherin colocalization (6 days-treatment) and eventually absence
11 of both, epithelial Dab2 and E-cadherin at 9 days-treatment.
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17 Human inflamed colon and polyps exhibited increased Dab2/E-cadherin
18 colocalization but progression to adenocarcinoma is accompanied either by loss of
19 epithelial Dab2 and delocalization of E-cadherin. Therefore, as in rodents, human colon
20 initially responds to damage by increasing Dab2/E-cadherin colocalization. In control
21 conditions Dab2/E-cadherin colocalization is mainly observed at the bottom of the
22 crypts, where the stem cells are located. Once the new cells established E-cadherin-
23 mediated contacts, cell proliferation is stopped (see Mendonsa, Na, & Gumbiner, 2018
24 for review). Hence, by contributing to the proper E-cadherin location at the cell-cell
25 junction, Dab2 might regulate cell proliferation/differentiation transition. The
26 observations made in Caco-2 cells corroborate this point of view and all together, the
27 current findings are consistent with a role of Dab2 in the proper E-cadherin location at
28 the cell-cell junction, thus maintaining the junction stability and the intestinal epithelium
29 integrity and cell differentiation. Thus, Dab2 down-regulation produces loss of epithelial
30 polarity during embryo development and carcinogenesis (Moore, Cai, Tao, Smith, &
31 Xu, 2013; Yang et al., 2007, 2006, 2002) and links to the process of EMT (Chao et al.,
32 2012; Hocevar, 2019; Martin et al., 2010; Piao et al., 2017). This role of Dab2 in
33 epithelial polarity has been proposed as an underlying mechanism for its tumour-
34 suppressive function (see Finkielstein & Capelluto, 2016 for review).
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46 Progression of mice and human colon disorders, here studied, led to the
47 appearance of non-epithelial cells expressing Dab2, which might represent immune
48 cells, such as macrophages and lymphocytes. Upregulation of Dab2 in immune cells
49 has been found under inflammatory conditions in other tissues (Adamson et al., 2016;
50 Ahmed et al., 2015; Ahn et al., 2015; Jokubaitis et al., 2013; Moon, Lee, Ahn, & Shin,
51 2005).
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55 In conclusion, as far as we know, this is the first report showing localization of
56 Dab2 at the intestinal epithelium junctions *ex vivo* and that its specific depletion in
57 culture cells led to E-cadherin internalization. Furthermore, our colitis model and
58 pathological human tissue results also suggest a functional link between Dab2 and E-
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3 cadherin and that Dab2 might exert a protective function against mice colitis and
4 human ulcerative colitis and cancer. Further immunoprecipitation assays of Dab2 with
5 junctional proteins, such as E-cadherin, or with endo/exocytosis machinery-related
6 proteins would help unravel the mechanism/s underlying Dab2-mediated proper E-
7 cadherin location. Deciphering the mechanisms that control epithelial Dab2 expression
8 would tell whether Dab2 could be used as a biomarker for diagnosis and prognosis of
9 colon inflammation and cancer and as a therapeutic target in the future.
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15 **CONFLICT OF INTERESTS**

16 The authors declare that there are no conflicts of interests.
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20 **DATA AVAILABILITY STATEMENT**

21 The data that support the findings of this study are available from the corresponding
22 author upon reasonable request.
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For Peer Review

Legends

FIGURE 1 Immunolocalization of Dab2 in the ileum and colon of suckling (8-day-old) and adult (3-month-old) mice. 5 μm tissue sections were incubated with anti-Dab2 (Santa Cruz, red) antibody. Dab2 localization at apical membrane (arrowheads) or at the junctions (small arrows) are pointed. Scale bars represent 50 μm . The photographs are representative of five different assays for each condition. See Materials and Methods for specific details.

FIGURE 2 Effects of Dab2 depletion on expression and location of tight- and adherens-junctions proteins. Caco-2 cells transfected with either non-targeting (control) or siRNAs directed against Dab2 (siDab2) were used. (a) Effect of Dab2 silencing on the Dab2 protein abundance analysed by Western blot; an anti-Dab2 antibody (BD Transduction Laboratories) was used, (b) immunofluorescence and (c) Western-blots using antibodies against the indicated proteins. α -tubulin was used as standard. Histograms represent the relative abundance of protein. The values represent means \pm SEM of arbitrary units of protein abundance, Student's *t* test: * $p < 0,05$ vs control. Scale bars represent 10 μm . The photographs are representative of five different assays. See Materials and Methods for specific details.

FIGURE 3 Immunolocalization of Dab2 and E-cadherin in the ileum and colon of suckling (8-day-old) and adult (3-month-old) mice. 5 μm tissue sections were incubated with anti-Dab2 (Santa Cruz, red) and anti-E-cadherin (green) antibodies. Nuclei were visualized with Hoechst (blue). Dab2 localization at the apical membrane (arrowheads) or at the junctions (small arrows) are pointed. Yellow-orange colour indicates colocalization of both proteins (pointed with large arrows). Scale bars represent 50 μm . The photographs are representative of five different assays. See Materials and Methods for specific details.

FIGURE 4 Effect of inflammation on Dab2 and E-cadherin location in the mice colon. 5 μm colon sections of 3 month-old mice without and with 3, 6 or 9 days DSS-treatment were incubated with anti-Dab2 (Santa Cruz, red) and anti-E-cadherin (green) antibodies. Nuclei were visualized with Hoechst (blue). Yellow-orange colour indicates colocalization of the both proteins under study (pointed with arrows). (a) Representative photographs of five different immunohistochemistry assay. Scale bars represent 50 μm . Histograms represent means \pm SEM of (b) Dab2 or E-cadherin immunofluorescence intensity per crypt and (c) each bar represents the total number of cells per crypt and the number of cells having Dab2/E-cadherin colocalization per crypt.

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3 One-way ANOVA showed an effect of inflammation progression on Dab2 and E-
4 cadherin immunofluorescence intensity and Dab2/E-cadherin colocalization per crypt
5 ($p < 0.01$). Tukey's test: ^a $p < 0.01$, DSS-treated vs untreated mice; ^b $p < 0.01$, 6 days DSS-
6 treated vs 3 days DSS-treated mice. Five mice were used per condition. See Materials
7 and Methods for specific details.
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12 **FIGURE 5** Immunolocalization of Dab2 and E-cadherin in human colon under
13 inflammation conditions. 5-10 μm colon sections from human specimens of: healthy
14 colon, mild ulcerative colitis and severe ulcerative colitis, were incubated with anti-
15 Dab2 (Proteintech, red) and anti-E-cadherin (green) antibodies. Nuclei were visualized
16 with Hoechst (blue). Yellow-orange colour indicates colocalization of the both proteins
17 under study (pointed with arrows). Representative photographs of the
18 immunohistochemistry assay are shown. Scale bars represent 50 μm . Five specimens
19 were used per condition. See Materials and Methods for specific details.
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25 **FIGURE 6** Immunolocalization of Dab2 and E-cadherin in human colon polyps and
26 adenocarcinomas. 5-10 μm colon sections from human specimens of: non-
27 adenomatous polyps and adenocarcinoma were incubated with anti-Dab2 (Proteintech,
28 red) and anti-E-cadherin (green) antibodies. Nuclei were visualized with Hoechst
29 (blue). Dab2 localization at the membrane (arrowheads) or at the cytosol (small arrows)
30 are pointed. Yellow-orange colour indicates colocalization of the both proteins under
31 study (pointed with large arrows). Scale bars represent 50 μm . Representative
32 photographs of the immunohistochemistry assay are shown. Healthy colon is shown in
33 Figure 5. Adenocarcinomas labelled as 1 or 2 indicate two different representative
34 areas within the same adenocarcinoma specimen. Five specimens were used per
35 condition. See Materials and Methods for specific details.
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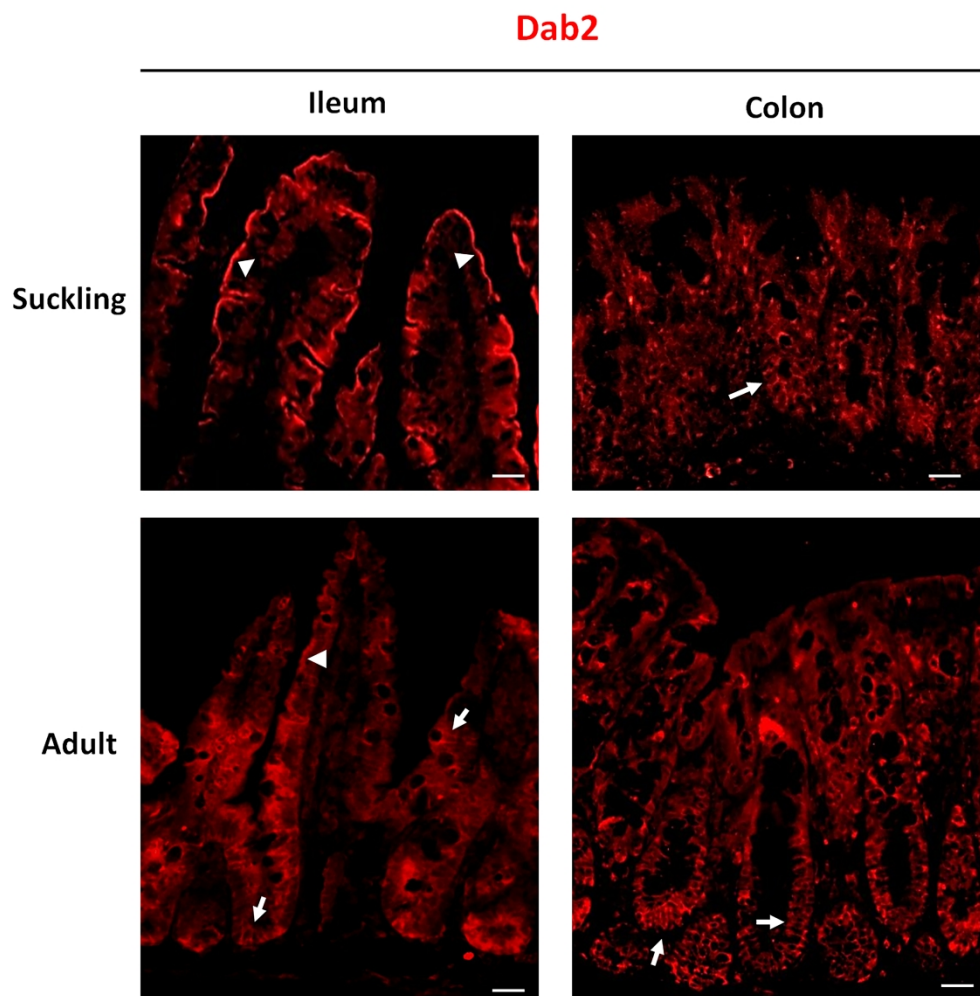
44 **FIGURE 7** Quantification of immunofluorescence intensity and Dab2/E-cadherin
45 colocalization in human colon specimens. Histograms represent means \pm SEM. (a)
46 Dab2 or E-cadherin immunofluorescence intensity per crypt and (b) each bar
47 represents the total number of cells per crypt and the number of cells having Dab2/E-
48 cadherin colocalization per crypt. UC: ulcerative colitis. One-way ANOVA showed an
49 effect of inflammation progression on Dab2 and E-cadherin immunofluorescence
50 intensity and Dab2/E-cadherin colocalization per crypt ($p < 0.01$). Tukey's test: ^a $p < 0.01$,
51 UC vs healthy colon; ^b $p < 0.01$, severe UC vs mild UC or polyps vs mild UC. Five
52 specimens were used per condition. See Materials and Methods for specific details.
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3 **SUPPLEMENTARY FIGURE 1** Evaluation of the DSS-induced colitis progression. DSS
4 (dextran sulphate sodium) was administered in the drinking water during either 3, 6 or 9
5 days as described in Methods. The following inflammation indicators are displayed:
6 DAI, histological score of colon, representative photographs of H&E stained distal colon
7 sections and mRNA relative abundance of IL-1 β and TNF- α . Scale bars represent 100
8 μ m. Data are means \pm SEM. The number of animals used in each experimental
9 condition was 5. Student's *t*-test: * p <0.001, ** p <0.05, DSS-treated vs. untreated mice.
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15 **SUPPLEMENTARY FIGURE 2** Negative controls of immunofluorescence assays. 5-10
16 μ m ileum and colon sections from either mouse or patient specimens used in figures 1
17 and 3-6 were incubated without primary antibody but with the anti-rabbit IgG Alexa
18 fluor-546 secondary antibody (Life technologies). Scale bars represent 50 μ m. The
19 photographs are representative of five different assays.
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24 **SUPPLEMENTARY FIGURE 3** Effects of Dab2 depletion on paracellular and ion
25 permeability. (a) Paracellular tracer permeability was measured on Caco-2 cells using
26 fluorescently labelled dextrans of 4 and 70 kDa. (b) Transepithelial Electrical
27 Resistance (TER) was measured on Caco-2 cells for a period of 24, 30, 48 and 60
28 hours culture. Data are means \pm SEM, n =3.
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33 **SUPPLEMENTARY FIGURE 4** Immunolocalization of Dab2 and arginase-1. The
34 colons of 9 days DSS-treated mice and of the human specimens (severe ulcerative
35 colitis or adenocarcinoma) were utilized. 5-10 μ m sections of the specimens were
36 incubated with anti-Dab2 (either Santa Cruz for mouse or Proteintech for human
37 samples, red) and anti-arginase-1 (green) antibodies. Nuclei were visualized with
38 Hoechst (blue). Yellow-orange colour indicates colocalization of the both proteins under
39 study (pointed with arrows). Scale bars represent 50 μ m. The photographs are
40 representative of five different assays.
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39 **FIGURE 1** Immunolocalization of Dab2 in the ileum and colon of suckling (8-day-old) and adult (3-month-
40 old) mice. 5 μm tissue sections were incubated with anti-Dab2 (Santa Cruz, red) antibody. Dab2 localization
41 at apical membrane (arrowheads) or at the junctions (small arrows) are pointed. Scale bars represent 50
42 μm . The photographs are representative of five different assays for each condition. See Materials and
43 Methods for specific details.

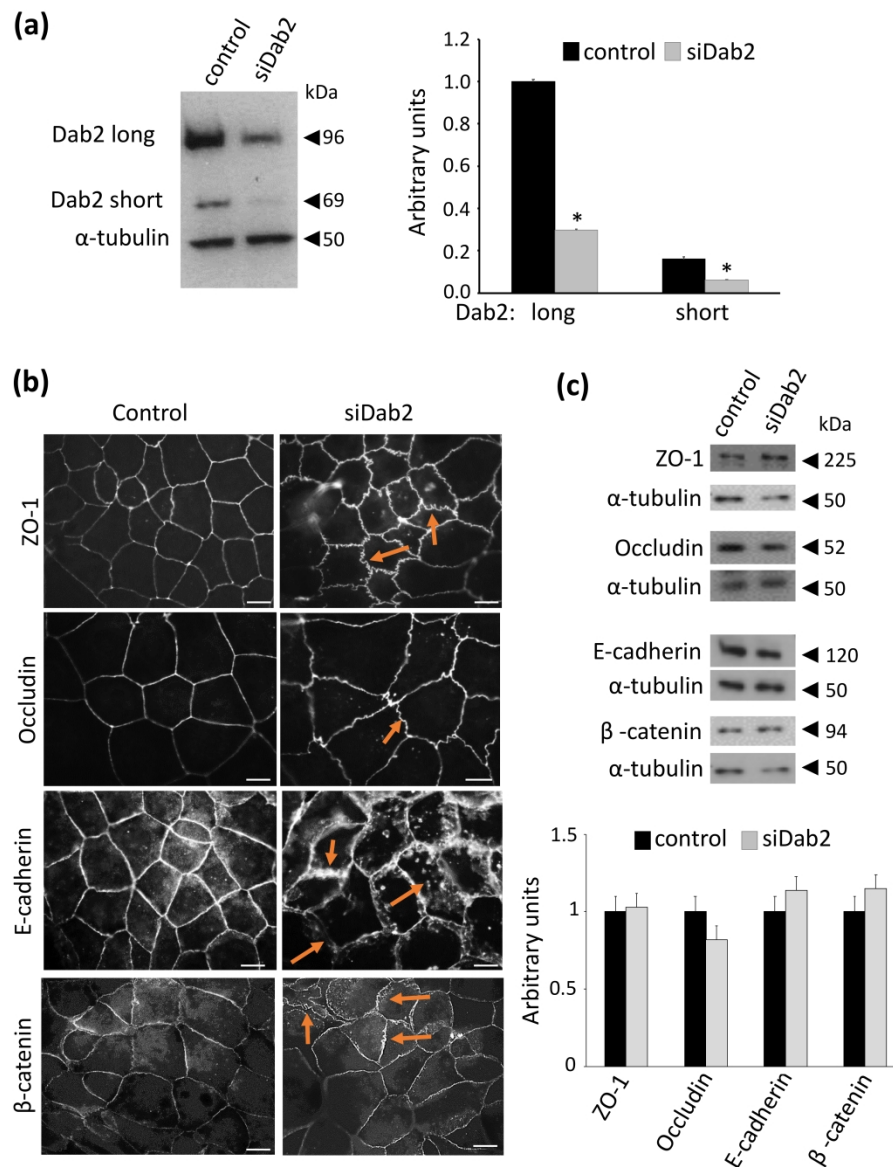
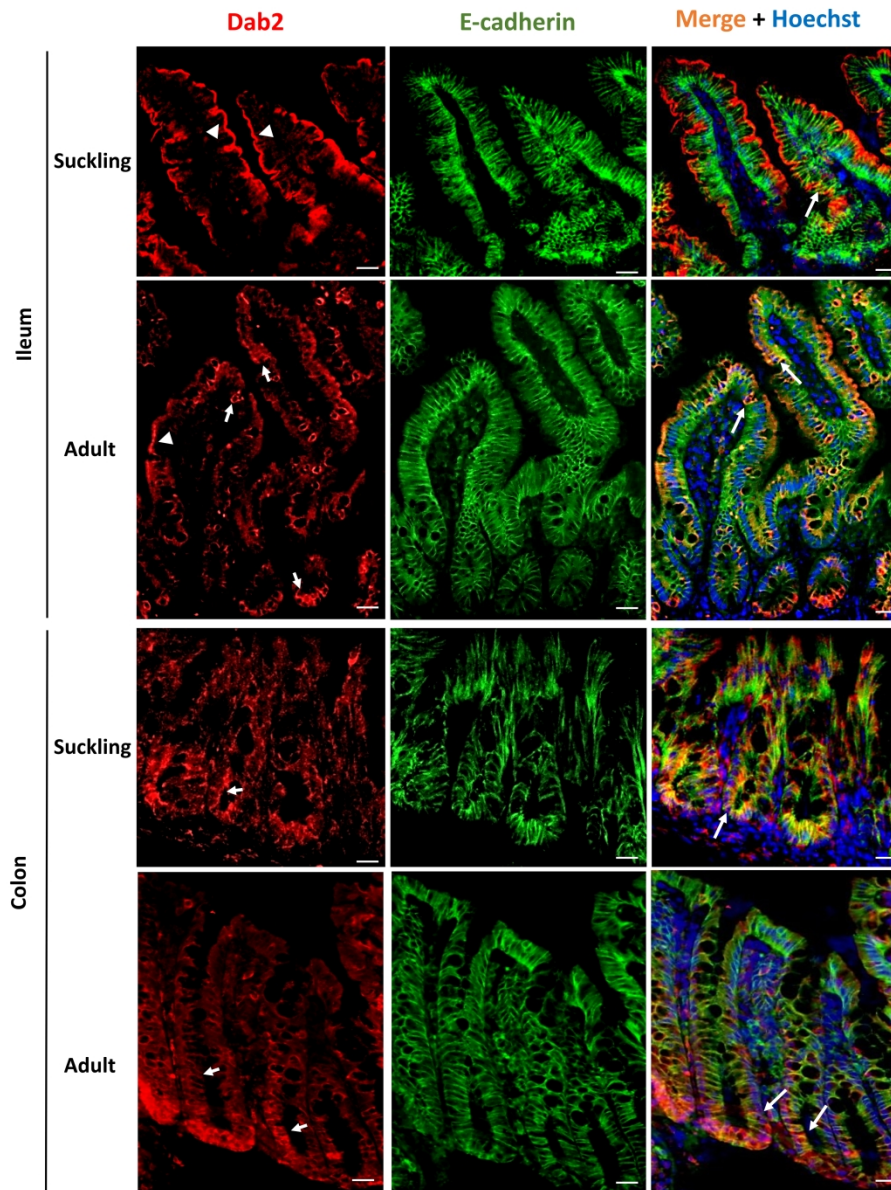


FIGURE 2 Effects of Dab2 depletion on expression and location of tight- and adherens- junctions proteins. Caco-2 cells transfected with either non-targeting (control) or siRNAs directed against Dab2 (siDab2) were used. (a) Effect of Dab2 silencing on the Dab2 protein abundance analysed by Western blot; an anti-Dab2 antibody (BD Transduction Laboratories) was used, (b) immunofluorescence and (c) Western-blot using antibodies against the indicated proteins. α -tubulin was used as standard. Histograms represent the relative abundance of protein. The values represent means \pm SEM of arbitrary units of protein abundance, Student's t test: * $p < 0,05$ vs control. Scale bars represent 10 μ m. The photographs are representative of five different assays. See Materials and Methods for specific details.

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FIGURE 3 Immunolocalization of Dab2 and E-cadherin in the ileum and colon of suckling (8-day-old) and adult (3-month-old) mice. 5 μ m tissue sections were incubated with anti-Dab2 (Santa Cruz, red) and anti-E-cadherin (green) antibodies. Nuclei were visualized with Hoechst (blue). Dab2 localization at the apical membrane (arrowheads) or at the junctions (small arrows) are pointed. Yellow-orange colour indicates colocalization of both proteins (pointed with large arrows). Scale bars represent 50 μ m. The photographs are representative of five different assays. See Materials and Methods for specific details.

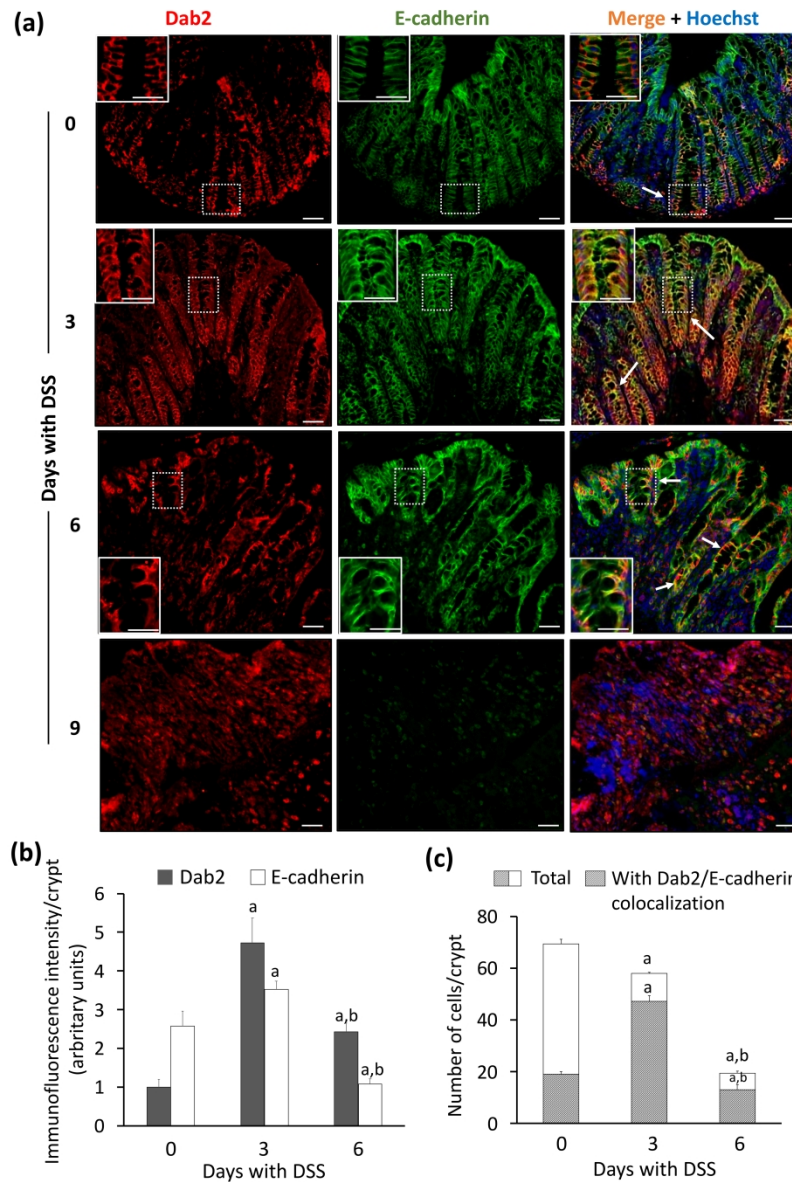
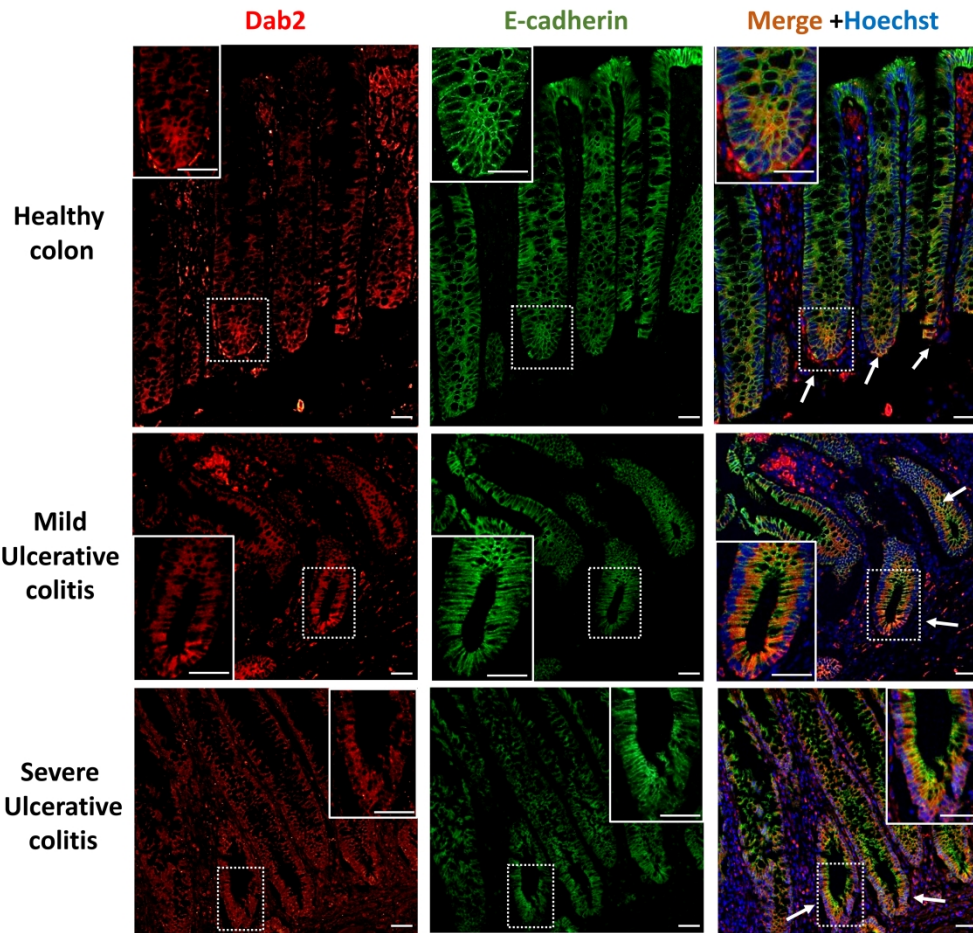
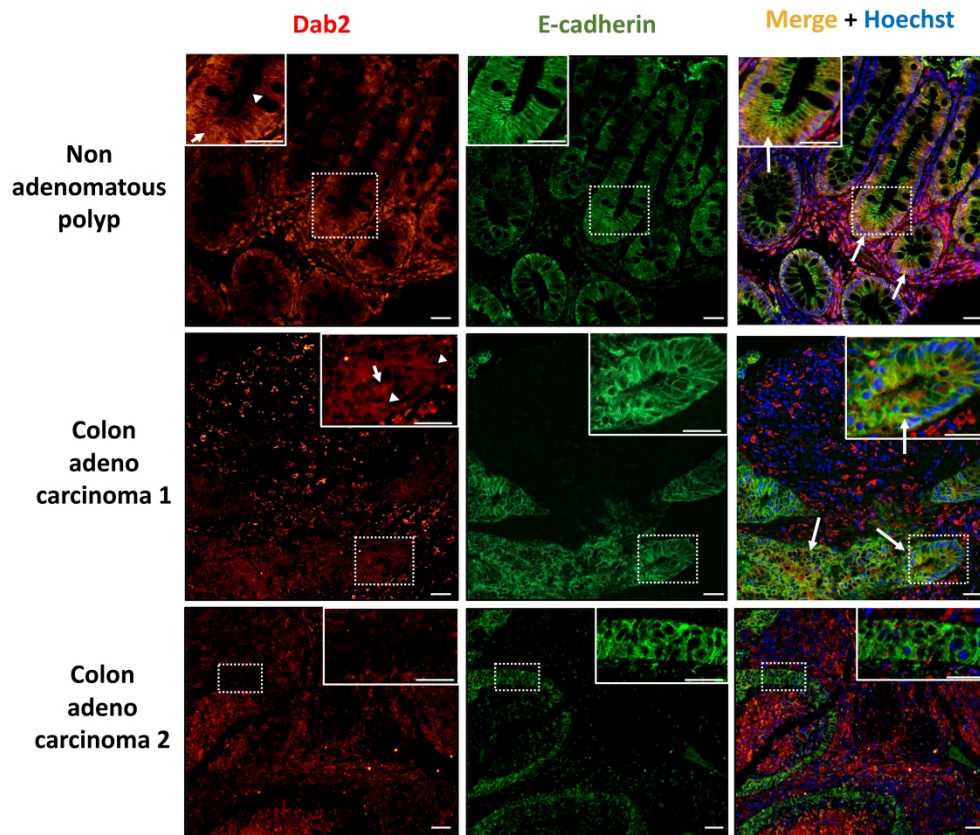


FIGURE 4 Effect of inflammation on Dab2 and E-cadherin location in the mice colon. 5 μm colon sections of 3 month-old mice without and with 3, 6 or 9 days DSS-treatment were incubated with anti-Dab2 (Santa Cruz, red) and anti-E-cadherin (green) antibodies. Nuclei were visualized with Hoechst (blue). Yellow-orange colour indicates colocalization of the both proteins under study (pointed with arrows). (a) Representative photographs of five different immunohistochemistry assay. Scale bars represent 50 μm . Histograms represent means \pm SEM of (b) Dab2 or E-cadherin immunofluorescence intensity per crypt and (c) each bar represents the total number of cells per crypt and the number of cells having Dab2/E-cadherin colocalization per crypt. One-way ANOVA showed an effect of inflammation progression on Dab2 and E-cadherin immunofluorescence intensity and Dab2/E-cadherin colocalization per crypt ($p < 0.01$). Tukey's test: ^a $p < 0.01$, DSS-treated vs untreated mice; ^b $p < 0.01$, 6 days DSS-treated vs 3 days DSS-treated mice. Five mice were used per condition. See Materials and Methods for specific details.



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FIGURE 5 Immunolocalization of Dab2 and E-cadherin in human colon under inflammation conditions. 5-10 μm colon sections from human specimens of: healthy colon, mild ulcerative colitis and severe ulcerative colitis, were incubated with anti-Dab2 (Proteintech, red) and anti-E-cadherin (green) antibodies. Nuclei were visualized with Hoechst (blue). Yellow-orange colour indicates colocalization of the both proteins under study (pointed with arrows). Representative photographs of the immunohistochemistry assay are shown. Scale bars represent 50 μm . Five specimens were used per condition. See Materials and Methods for specific details.



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FIGURE 6 Immunolocalization of Dab2 and E-cadherin in human colon polyps and adenocarcinomas. 5-10 μm colon sections from human specimens of: non-adenomatous polyps and adenocarcinoma were incubated with anti-Dab2 (Proteintech, red) and anti-E-cadherin (green) antibodies. Nuclei were visualized with Hoechst (blue). Dab2 localization at the membrane (arrowheads) or at the cytosol (small arrows) are pointed. Yellow-orange colour indicates colocalization of the both proteins under study (pointed with large arrows). Scale bars represent 50 μm . Representative photographs of the immunohistochemistry assay are shown. Healthy colon is shown in Figure 5. Adenocarcinomas labelled as 1 or 2 indicate two different representative areas within the same adenocarcinoma specimen. Five specimens were used per condition. See Materials and Methods for specific details.

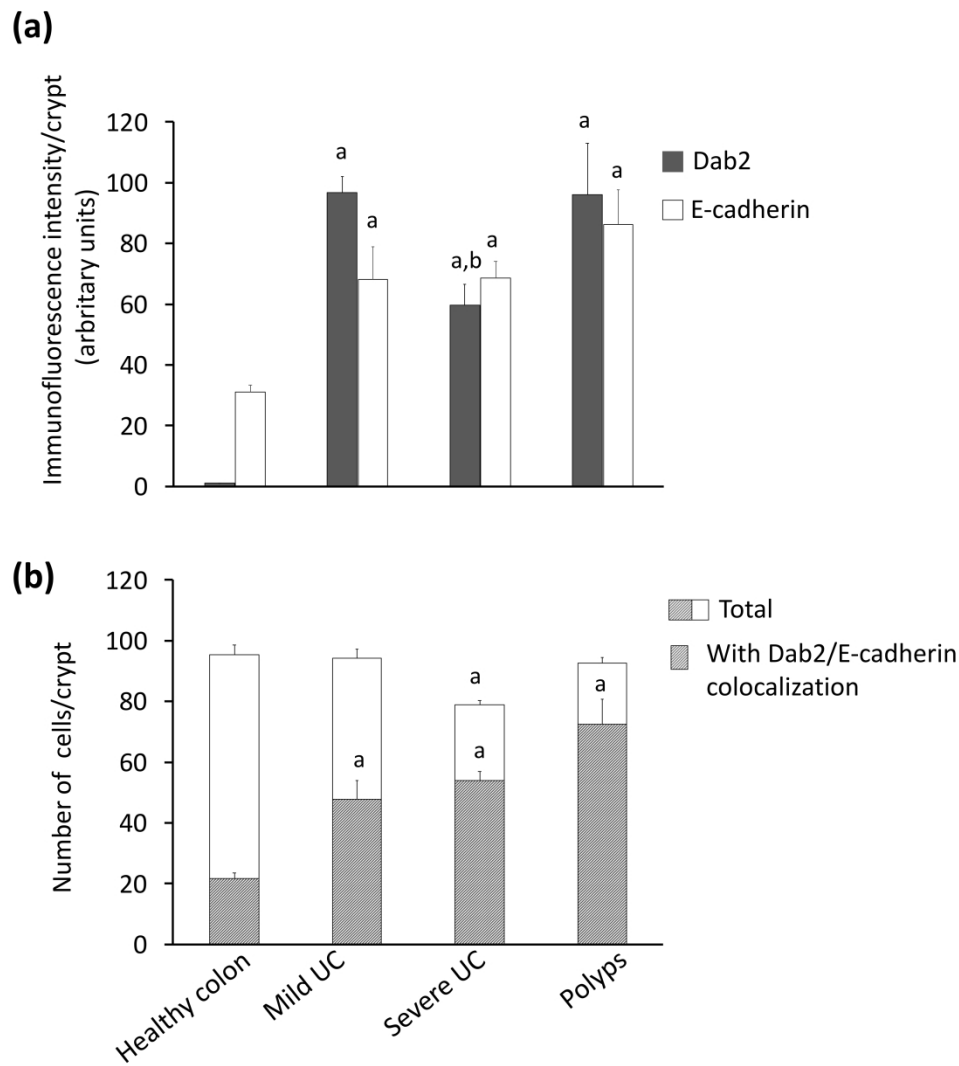


FIGURE 7 Quantification of immunofluorescence intensity and Dab2/E-cadherin colocalization in human colon specimens. Histograms represent means \pm SEM. (a) Dab2 or E-cadherin immunofluorescence intensity per crypt and (b) each bar represents the total number of cells per crypt and the number of cells having Dab2/E-cadherin colocalization per crypt. UC: ulcerative colitis. One-way ANOVA showed an effect of inflammation progression on Dab2 and E-cadherin immunofluorescence intensity and Dab2/E-cadherin colocalization per crypt ($p < 0.01$). Tukey's test: ^a $p < 0.01$, UC vs healthy colon; ^b $p < 0.01$, severe UC vs mild UC or polyps vs mild UC. Five specimens were used per condition. See Materials and Methods for specific details.