


ORIGINAL RESEARCH ARTICLE

Dual-specificity phosphatase 6 deletion protects the colonic epithelium against inflammation and promotes both proliferation and tumorigenesis

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Abstract

The Ras/mitogen-activated protein kinase (MAPK) pathway controls fundamental cellular processes such as proliferation, differentiation, and apoptosis. The dual-specificity phosphatase 6 (DUSP6) regulates cytoplasmic MAPK signaling by dephosphorylating and inactivating extracellular signal-regulated kinase (ERK1/2) MAPK. To determine the role of DUSP6 in the maintenance of intestinal homeostasis, we characterized the intestinal epithelial phenotype of *Dusp6* knockout (KO) mice under normal, oncogenic, and proinflammatory conditions. Our results show that loss of *Dusp6* increased crypt depth and epithelial cell proliferation without altering colonic architecture. Crypt regeneration capacity was also enhanced, as revealed by ex vivo *Dusp6* KO organoid cultures. Additionally, loss of *Dusp6* induced goblet cell expansion without affecting enteroendocrine and absorptive cell differentiation. Our data also demonstrate that *Dusp6* KO mice were protected from acute dextran sulfate sodium-induced colitis, as opposed to wild-type mice. In addition, *Dusp6* gene deletion markedly enhanced tumor load in *Apc*^{Min/+} mice. Decreased DUSP6 expression by RNA interference in HT29 colorectal cancer cells enhanced ERK1/2 activation levels and promoted both anchorage-independent growth in soft agar as well as invasion through Matrigel. Finally, *DUSP6* mRNA expression in human colorectal tumors was decreased in advanced stage tumors compared with paired normal tissues. These results demonstrate that DUSP6 phosphatase, by controlling ERK1/2 activation, regulates colonic inflammatory responses, and protects the intestinal epithelium against oncogenic stress.

KEYWORDS

colitis, colorectal cancer, *Dusp6*, ERK, intestinal epithelium, proliferation

Abbreviations: APC, adenomatous polyposis coli; CRC, colorectal cancer; DSS, dextran sulfate sodium; DUSP, dual-specificity phosphatase; EdU, 5-ethynyl-2-deoxyuridine; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; KRAS, Kirsten rat sarcoma viral oncogene homolog; IEC, intestinal epithelial cells; MAPK, mitogen-activated protein kinase; MEK, MAPK/Erk kinase; MKP, mitogen-activated protein kinase phosphatase; Min, multiple intestinal neoplasia; MSI, microsatellite instability; MSS, microsatellite stable; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; shRNA, short hairpin RNA; TGF, transforming growth factor; Wnt, wingless.

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1 | INTRODUCTION

The intestinal epithelium is constantly renewed and repaired throughout life by the action of multipotent stem cells that give rise to a population of proliferative and undifferentiated cells. These progenitor cells, also called transit-amplifying (T/A) cells, proliferate rapidly while migrating along the crypt axis. In the small intestine, the progenitor cells cease their proliferation after two to three divisions and differentiate in absorptive enterocytes, mucus-producing goblet cells, hormone-secreting enteroendocrine cells, or Paneth cells. While the first three cell types differentiate during an upward migration from the crypt to the adjacent villus, Paneth cells differentiate during a downward movement to the crypt base. In contrast to the small intestine, the colonic epithelium displays epithelial caps instead of villi and deeper crypts devoid of Paneth cells (Barker, 2014; Crosnier, Stamatakis, & Lewis, 2006).

A central role for wingless (Wnt), bone morphogenetic proteins/transforming growth factor β (TGF β), Notch, and epidermal growth factor (EGF) signal transduction pathways in the maintenance, proliferation, and differentiation of intestinal stem and progenitor cells has been demonstrated in several murine models (Andreu et al., 2005; Carragher et al., 2010; van Es et al., 2005; Feng et al., 2011; Fre et al., 2005; Haigis et al., 2008; Haramis et al., 2004; He et al., 2004; Janssen et al., 2002; Jensen et al., 2000; Korinek et al., 1998; Kuhnert et al., 2004; Milano et al., 2004; Pinto, Gregorieff, Begthel, & Clevers, 2003; Sansom et al., 2004; Yang, Bermingham, Finegold, & Zoghbi, 2001; Zecchini, Domaschek, Winton, & Jones, 2005). EGF and its orthologs epiregulin and TGF α , control epithelial cell proliferation by activating the Ras/Raf/MEK/ERK (extracellular signal-regulated kinase) mitogen-activated protein kinase pathway (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (Riese & Cullum, 2014; Wee & Wang, 2017). More specifically, we and others have observed a close correlation between the activation of MAPK/Erk kinase (MEK)/ERK signaling and proliferation on cultured intestinal epithelial cells (IEC; Aliaga, Deschênes, Beaulieu, Calvo, & Rivard, 1999; Boucher, Jean, Vézina, & Rivard, 2004; Dionne et al., 1998; Paquin, Cagnol, Carrier, Leblanc, & Rivard, 2013; Rhoads et al., 1997; Rivard, Boucher, Asselin, & L'Allemain, 1999; Sheng, Bernabe, Guo, & Warner, 2006). Indeed, pharmacological inhibition of MEK activity blocks IEC cell cycle progression in G1 phase (Aliaga et al., 1999; Balmanno, Chell, Gillings, Hayat, & Cook, 2009; Paquin et al., 2013; Rivard et al., 1999). Accordingly, high ERK activity is detected in progenitor cells of the crypt T/A zone in human intestinal epithelium where ERK1/2 may direct the proliferation to differentiation switch (Aliaga et al., 1999). Indeed, we and others have reported that ERK1/2 are selectively inactivated during absorptive cell differentiation (Aliaga et al., 1999; Ding, Wang, & Evers, 2001; Lemieux et al., 2011; Taupin & Podolsky, 1999). These data support the hypothesis that ERK1/2 kinase activity must be shut down to initiate the differentiation process in the intestine, at least in enterocytes.

ERK1/2 activation depends on phosphorylation of threonine and tyrosine amino acids in a specific TEY motif. Dephosphorylation of these residues by specific dual-specificity phosphatases (DUSP), also known as mitogen-activated protein kinase phosphatases

(MKP), mediates the end of the signal. There are two groups of ERK1/2-targeting DUSP depending on their subcellular localization, namely, DUSP1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2, and DUSP5 localized in the nucleus, or DUSP6/MKP-3, DUSP7/MKP-X, and DUSP9/MKP-4 localized in the cytoplasm (Kidger & Keyse, 2016; Owens & Keyse, 2007). DUSPs are characterized by their variable N-terminal MAPK-binding region including the kinase interaction motif (KIM) that governs substrate specificity and stability of interaction (Owens & Keyse, 2007; Tanoue, Adachi, Moriguchi, & Nishida, 2000). Very few studies have analyzed the expression or function of these enzymes in the intestine. Interestingly, the elevated DUSP1 expression is detected in differentiated villus cells but not in proliferating crypt cells in the adult intestine (Noguchi et al., 1993). In the mouse intestine, *Dusp6* is predominantly detected in the differentiated epithelium (Ruan et al., 2016). In line with these results, expression of both DUSP1 and DUSP6 markedly increases during enterocyte differentiation of human Caco-2/15 cells, hence correlating with dramatic inhibition of ERK1/2 activities (Aliaga et al., 1999; Taupin & Podolsky, 1999). These observations suggest that ERK activity is tightly controlled by DUSP, both in its duration and intensity, during IEC differentiation.

In the current study, we have focused on the role of DUSP6, the first MKP to be characterized with absolute substrate specificity for ERK as opposed to either JNK or p38 (Groom, Sneddon, Alessi, Dowd, & Keyse, 1996; Muda et al., 1996). More specifically, this phosphatase recognizes and binds the biphosphorylated ERK (pT183pY185) via its conserved KIM domain. Binding to ERK1/2 allosterically increases the catalytic activity of DUSP6 which then dephosphorylates ERK1/2 (Camps et al., 1998).

To determine the role of DUSP6 in intestinal homeostasis, we have analyzed the intestinal epithelial phenotype of *Dusp6* knockout (KO) mice under normal, oncogenic, and proinflammatory conditions. Our results demonstrate that DUSP6, by controlling ERK1/2 activation, regulates colonic inflammatory responses and protects the intestinal epithelium against oncogenic stress.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Primary antibodies were obtained from the following sources: DUSP6 (ab76310) from Abcam (Toronto, Canada), β -actin (MAB1501R) from Millipore (Oakville, Canada), ERK2 (sc-154) and MEK2 (sc-524) from Santa Cruz Biotechnology (Santa Cruz, CA), phosphorylated ERK1/2 (M8159) from Sigma-Aldrich (Oakville, Canada), phosphorylated MEK1/2 (9121) from Cell Signaling Technology (Danvers, MA, USA), chromogranin A (20085) from ImmunoStar (Hudson, WI), proliferating cell nuclear antigen (PCNA; 18197) from Abcam and Ki67 (GTX16667) from Genetex (Irvine, CA). Horseradish peroxidase antibodies were obtained from GE Healthcare Life Sciences (Mississauga, Canada) and alkaline phosphatase-conjugated antibodies from Promega (Madison, WI). For immunofluorescence, Alexa Fluor 488 conjugated antibodies were obtained from

Molecular Probes (Waltham, MA). Other materials were purchased from Sigma-Aldrich unless stated otherwise.

2.2 | Animals

Dusp6^{+/-} mice were obtained from Jefferey D. Molkenin (Cincinnati, OH; Maillet et al., 2008) and C57BL6/J-*Apc*^{Min/+} mice were purchased from Jackson Laboratory. *Dusp6*^{+/-} mice were bred to generate *Dusp6*^{-/-} experimental mice and *Dusp6*^{+/+} control littermates. For tumor initiation experiments, *Apc*^{Min/+} mice were crossed with *Dusp6*^{+/-} mice to obtain double heterozygous *Dusp6*^{+/-};*Apc*^{Min/+} mice. *Dusp6*^{+/-};*Apc*^{Min/+} mice were then bred with *Dusp6*^{+/-} mice to obtain experimental *Dusp6*^{-/-};*Apc*^{Min/+} mice and *Dusp6*^{+/+};*Apc*^{Min/+} control littermates. For genotyping, genomic DNA was extracted with a 25 mM NaOH/0.2 mM ethylenediaminetetraacetic acid (EDTA) solution heated at 95°C for 1 hr, followed by addition of an equal volume of 40 mM Tris-HCl (pH 5.5). Primer sequences and polymerase chain reaction (PCR) conditions are available upon request. All experiments were approved by the Animal Research Ethics Committee of the Faculty of Medicine and Health Sciences of the Université de Sherbrooke (Protocols: 348-13B and 348-18B).

2.3 | Macroadenomas count, histological staining, immunofluorescence, and immunohistochemistry

Methylene blue-stained polyps were visualized under an SZ51 stereomicroscope (Olympus, Richmond Hill, Canada). Polyp sizes were measured with a digital caliper (Thermo Fisher Scientific, Waltham, MA) and polyp numbers were counted from the duodenum to the rectum as previously described (Perreault, Sackett, Katz, Furth, & Kaestner, 2005). Tissues were fixed, paraffin embedded, sectioned, and stained as described before (Leblanc et al., 2017). Immunohistochemistry staining was performed with the Dako EnVision+System Kit (Dako, Santa Clara, CA) according to the manufacturer's recommendations. Immunofluorescence against chromogranin A was performed as described previously (C. S. Lee, Perreault, Brestelli, & Kaestner, 2002). Mucus secretion was visualized by Alcian blue staining performed on distal colon tissues fixed with Carnoy's solution (10% glacial acetic acid, 30% chloroform, and 60% ethanol). For immunofluorescence, images were taken with a Leica DLMB2 microscope equipped with a DFC300FX camera and Leica FireCAM 3.4.1 Software (Leica, Concord, Canada). Otherwise, slides were visualized with a NanoZoomer slide scanner and NDP.view2 software (Hamamatsu, Boston, MA). All cell counts were performed on well-oriented crypts in a double-blind manner.

2.4 | Colonoid culture

Twelve-week-old murine colons were cut into 5-mm pieces and thoroughly washed in phosphate-buffered saline (PBS). Colon fragments were incubated for 1 hr in 5 mM EDTA/PBS solution. EDTA was replaced with PBS and colonic pieces were shaken vigorously until crypt dissociation. Crypts were then centrifuged at 350g for 3 min, and the pellet was washed twice with PBS before pellet resuspension in Matrigel (BD Corning, Corning, NY) and plating in a 48-well plate (Corning Costar;

Corning). Colonoids were cultured in Advanced DMEM/F-12 culture medium (Gibco, Waltham, MA) supplemented with 1 mM N-acetylcysteine, 50 ng/ml murine EGF (Life Technologies, Waltham, MA), B27 supplement 1× (Life Technologies), N2 supplement 1× (Life Technologies), 150 ng/ml recombinant Wnt3a (Abcam) in addition to 10% R-spondin 1 and 5% Noggin conditioned media. Noggin-Fc and R-spondin 1-Fc conditioned supernatants were recovered from HEK293T cell lines stably expressing R-spondin 1-Fc (Dr. C. Kuo, Stanford University, Stanford, CA) or Noggin-Fc (Dr. G. R. van den Brink, Hubrecht Institute, Utrecht, The Netherlands). Culture medium was changed every 2 days. Photos were taken at Days 5, 7, and 9 with an inverted Zeiss Axiovert 200M microscope (Zeiss, Toronto, Canada). Relative organoid areas were measured by encircling the periphery of each organoid in Image J software (NIH). Colonoid proliferation was evaluated by 5-ethynyl-29-deoxyuridine (EdU) incorporation for 1 hr, followed by 4% paraformaldehyde fixation and EdU detection with the Click-it EdU Alexa Fluor 555 Imaging Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Organoids were visualized by confocal microscopy with a Zeiss LSM 880 laser scan microscope using Zen Black software (Zeiss).

2.5 | Dextran sulfate sodium (DSS) treatment

Eleven-week-old mice were administered 2% DSS (36–50 kDa; MP Biomedicals) in drinking water ad libitum for 7 days. Disease activity index (DAI) was measured at sacrifice according to Cooper, Murthy, Shah, and Sedergran (1993) criteria: stool consistency (0–4), rectal bleeding (0–4), colon hardening (0–4), and occult bleeding (0–4). Histological score was based on the presence or absence of mucosal architecture destruction, immune cell infiltration, muscle thickening, Goblet cell depletion, and crypt abscesses, as previously described (Coulombe et al., 2016).

2.6 | Cell culture

HT29 and HCT116 colon carcinoma cell lines (ATCC, Manassas, VA) were grown in McCoy's medium containing 10% fetal bovine serum (FBS). The retroviral shDUSP6 pSUPERretro expression vector was obtained from Transat (Saint-Priest, France). Retroviruses were produced in HEK293T cells and were used to infect HT29 and HCT116 cells. Infected cells were selected with 2.5 µg/ml puromycin. For all experiments, at least three different cell populations originating from three different infections were analyzed.

2.7 | Soft agarose and invasion assays

Soft agarose assays were performed with 30,000 cells per well for HT29 cells and 15,000 cells per well for HCT116 cells as previously described (Bian et al., 2016). After 14 days, colonies were stained with MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 5 hr at 37°C. Images were acquired using an Infinity VX2 1100/26MX Imaging System (Vilber Lourmat, Marne-la-Vallée, France) and colonies were counted with the Image J software (NIH). Invasion assays were performed with 30,000 cells per insert, for both HT29 and HCT116 cells, in 24-well plate

Corning® BioCoat™ Matrigel® invasion chambers with 8- μ m polycarbonate membranes according to the manufacturer's procedure. Briefly, cells were seeded in media without serum and media containing 20% FBS was used as chemoattractant. After a 72 hr incubation for HT29 cells and 48 hr incubation for HCT116 cells, noninvasive cells were removed with two cotton swabs. Invading cells were fixed with methanol 100% and stained with crystal violet 1%. Invading cells were observed with a Leica DLMB2 microscope and the total number of cells per insert was manually counted.

2.8 | Human colorectal tissues

Samples from colorectal tumors and paired normal tissues, at least 10 cm from a tumor, were resected, processed, classified, and graded from patients who did not receive neoadjuvant therapy as previously described (Bian et al., 2016). Tissues were collected after obtaining the patient's written consent, according to the protocol approved by the Institutional Human Subject Review Board of the Centre Hospitalier Universitaire de Sherbrooke. Total RNA was extracted using the Totally RNA Kit (Invitrogen, Waltham, MA) and processed according to the manufacturer's instructions. Genomic DNA was extracted from formalin-fixed paraffin-embedded tissue using an FFPE DNA Isolation Kit for Cells and Tissues (Qiagen, Quebec City, Canada) and amplified by PCR. Presence of mutations in tumor samples was detected by direct sequencing by the Plateforme de Séquençage et de Génomique du Centre de Recherche du Centre Hospitalier de l'Université Laval, Quebec City, QC, Canada). Molecular characterization included microsatellite instability status (MSI panel markers: BAT25, BAT26, D5S346, D2S123, D17S250), oncogenic Kirsten rat sarcoma viral oncogene homolog (*KRAS*; codons 12, 13, and 61) and *BRAF* (V600E) mutations. Pathological and clinical data were obtained from medical records and are provided in Supporting Information Tables S1 and S2.

2.9 | Western blot analysis

Proteins were isolated from scraped colonic mucosa enrichments of 12-week-old mice in chilled radioimmunoprecipitation assay buffer. For HT29 and HCT116 cell cultures, protein samples were prepared in Laemmli buffer as previously described (Bian et al., 2016). Western blot analyses were performed as described previously (Bian et al., 2016).

2.10 | RNA extraction and quantitative real-time PCR (qPCR)

Mouse colonic RNA was extracted with the Qiagen RNeasy Kit according to the manufacturer's protocol, including on-column DNase digestion. Reverse transcription was performed with the Transcriptor reverse transcriptase (Roche, Mississauga, Canada) according to the manufacturer's instructions. Total RNA from human colorectal tissues was extracted with the Totally RNA Kit and reverse transcription was performed using AMV-RT (Roche) according to the manufacturer's protocol. Quantitative PCRs were performed by the

RNomics Platform of the Université de Sherbrooke. Target expression was quantified relatively to *Pum1*, *Psmc4*, and *Tbp* expression for murine tissues. *DUSP6* expression was normalized to *MRLP19*, *PUM1*, and *RPL13A* expression levels in human colorectal cancer (CRC) tissue samples. We have selected reference genes according to the library of about 10 genes (human and mouse) used by the RNomics Platform of the Université de Sherbrooke. The platform tested several reference genes and chose the three most stable ones with geNorm (<https://genorm.cmgg.be/>). Primer sequences and PCR conditions are available upon request.

2.11 | Statistical analyses

Statistics were calculated using Student's two-tailed *t* tests if not stated otherwise. Data are expressed as mean \pm SEM if not stated otherwise. Results were considered statistically significant at $*p \leq 0.05$ and $**p \leq 0.01$. Graphs and statistics were generated with GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Densitometric analyses were performed using Image J software (NIH).

3 | RESULTS

3.1 | *Dusp6* deletion promotes colonic epithelial cell proliferation

To characterize the role of *Dusp6* in the regulation of intestinal homeostasis, we compared wild-type *Dusp6*^{+/+} mice to *Dusp6*^{-/-} KO mice. *Dusp6*^{-/-} mice have been previously characterized and are viable and fertile (Maillet et al., 2008). Western blot analyses of colonic mucosal protein enrichments confirmed the loss of *Dusp6* expression in *Dusp6*^{-/-} mice, resulting in increased ERK1/2 phosphorylation levels, supporting ERK1/2 pathway excessive activation in the *Dusp6*-deficient colonic epithelium (Figure 1a). Histological analysis of *Dusp6*^{-/-} mouse colon sections did not reveal apparent alterations in colonic gland architecture except a significant increase in crypt depth (Figure 1b). In line with these observations, mutant mouse colons displayed increased proliferative cell numbers in comparison to control littermates, as assessed by Ki67 staining (Figure 1c). Of importance, increased proliferation was not a result of stem cell compartment expansion since expression of intestinal stem cell markers, namely, *Lgr5* and *Ascl2*, was not significantly modulated in *Dusp6*-deficient colons, as determined by qPCR analysis (Figure 1d).

To assess the intrinsic effect of *Dusp6* deficiency on intestinal epithelial cell regeneration, we used a crypt colonoid culture system. As shown in Figure 2a, loss of *Dusp6* significantly promoted colonic organoid development. Indeed, *Dusp6*^{-/-} colonoid sizes were larger after 7 and 9 days in culture compared to control colonoids (Figure 2b). Furthermore, *Dusp6*-deficient colonoid proliferation was increased in comparison to wild-type colonoids, as determined by EdU incorporation (Figure 2c). These data indicate that *Dusp6* deletion leads to increased IEC proliferation.

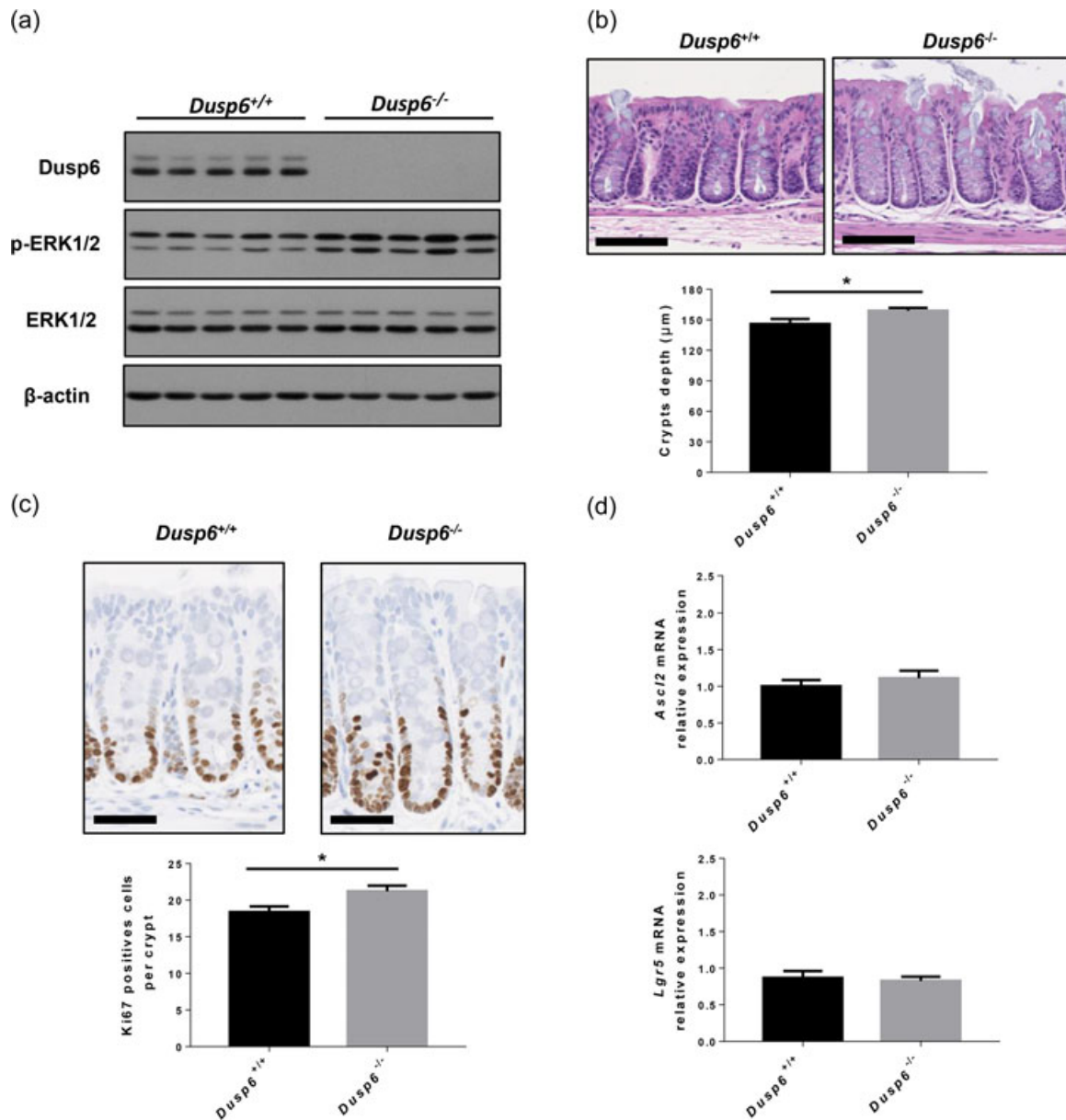


FIGURE 1 *Dusp6* deletion promotes colonic epithelial cell proliferation. (a) ERK1/2 phosphorylation and *Dusp6* expression were analyzed by western blot on colonic mucosal enrichments from 12-week-old *Dusp6* knockout and control mice ($n = 5$). β -Actin and ERK1/2 expression served as loading controls. Representative immunoblots are shown. (b) Colon tissue architecture was visualized with H&E staining and crypt depth was measured ($n = 11$). Scale bars=100 μ m. (c) Immunohistochemistry against Ki67 was performed to measure proliferation. The number of positive cells per crypt was counted ($n = 12$). Scale bars=50 μ m. (d) Relative *Ascl2* and *Lgr5* mRNA expression were quantified by qPCR with RNAs isolated from 12-week-old murine total colonic extracts ($n = 11$). Relative expression was normalized to housekeeping genes *Pum1*, *Tbp*, and *Psmc4* expression. Data are expressed as mean \pm SEM. Student's *t* test; * $p \leq 0.05$. DUSP6: dual-specificity phosphatase 6; ERK: extracellular signal-regulated kinase; H&E: hematoxylin and eosin; mRNA: messenger RNA; qPCR: quantitative polymerase chain reaction

3.2 | *Dusp6* deletion induces goblet cell expansion without affecting enteroendocrine and absorptive cell differentiation

We next verified whether loss of *Dusp6* modulates IEC differentiation in the colon. *Dusp6* deletion did not alter enteroendocrine cell differentiation. Indeed, the number of chromogranin-positive cells was not increased in mutant mouse colons in comparison to control mice, as assessed by immunofluorescence (Figure 3a). Additionally,

expression of the enteroendocrine cell marker *Neurogenin-3* was not significantly modified without *Dusp6* (Figure 3b). *Dusp6* deletion also did not alter absorptive cell differentiation as both control and mutant mouse colons exhibited similar expression level of the absorptive cell marker *Slc26a3*, as determined by qPCR (Figure 3b). Hence, *Dusp6* deletion does not affect enteroendocrine and absorptive cell differentiation.

In contrast, the number of goblet cells was significantly increased in *Dusp6*^{-/-} mouse colons in comparison to controls, as determined

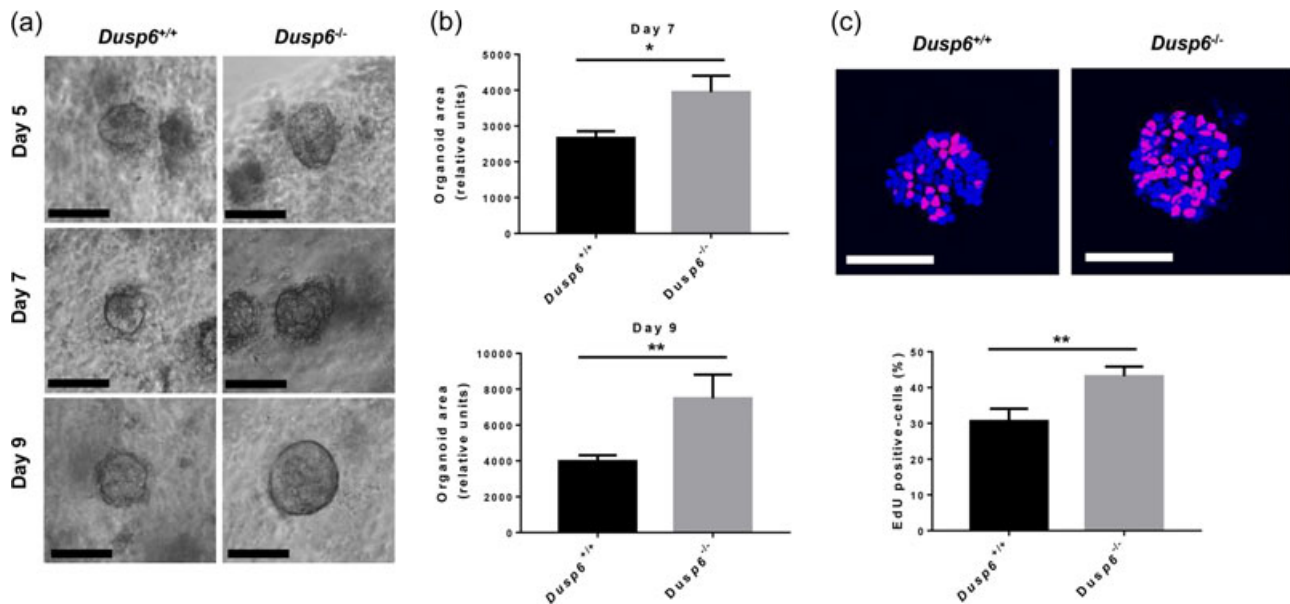


FIGURE 2 *Dusp6* deletion stimulates colonoid development. (a) Phase-contrast images of *Dusp6*^{+/+} and *Dusp6*^{-/-} colon organoids after 5, 7, and 9 days of culture. Scale bars: 100 μ m. (b) Relative organoid areas after 7 and 9 days of culture ($n \geq 33$). (c) EdU incorporation (red) and DAPI staining (blue) were used to evaluate the ratio of proliferative cells per organoid ($n = 16$). Scale bars: 50 μ m. Graphs are representative of at least three independent experiments conducted with different mice. Data are expressed as mean \pm SEM. Student's *t* test; * $p \leq 0.05$, ** $p \leq 0.01$. DAPI: 4',6-diamidino-2-phenylindole; DUSP6: dual-specificity phosphatase 6; EdU: 5-ethynyl-2-deoxyuridine

by Alcian blue staining which detects acidic mucins (Figure 3c). Notably, a thicker inner mucus layer was observed in *Dusp6*-deficient mouse colon lumen (Figure 3d), as evidenced by Carnoy's fixation. This indicates that increased colonic ERK-dependent signaling after *Dusp6* deletion selectively promotes goblet cell expansion and secretory function.

3.3 | *Dusp6* deletion protects against DSS-induced colitis in mice

We next assessed the susceptibility of *Dusp6*^{+/+} and *Dusp6*^{-/-} mice to colonic injury induced by DSS added in drinking water (Cooper et al., 1993). After 7 days, control mice had lost nearly 20% of their body weight (Figure 4a) and exhibited signs of diarrhea and rectal bleeding, resulting in high DAI (Figure 4b). In contrast, *Dusp6*^{-/-} mice treated with DSS exhibited less weight loss (Figure 4a), a decreased DAI associated with milder diarrhea and bloody stool symptoms (Figure 4b) and decreased histological alterations at the microscopic level (Figure 4c). These findings demonstrate that *Dusp6* inhibition protects the colon from DSS-induced acute injury.

3.4 | *Dusp6* deletion increases intestinal tumorigenesis in *Apc*^{Min/+} mice

Our data show that loss of *Dusp6* in IEC results in ERK signaling activation. We and others have shown previously that ERK is important for intestinal tumor development (Carragher et al., 2010; C. S. Lee, Perreault, et al., 2002; H.-W. Lee, Ahn, et al., 2002; Lemieux et al., 2009; Wang et al., 2013). To determine whether *Dusp6* deletion could affect

intestinal tumorigenesis, we crossed *Dusp6*^{-/-} mice with *Apc*^{Min/+} mice. These mice, heterozygous for an *Apc* truncated mutation frequently found in human sporadic CRC, spontaneously develop intestinal adenomas (Moser, Pitot, & Dove, 1990; Su et al., 1992). As shown in Figure 5a, *Dusp6* deficiency promoted intestinal tumor load in *Apc*^{Min/+} mice both in the small intestine and colon. Small intestinal tumors were also larger in *Dusp6*^{-/-}; *Apc*^{Min/+} mice as opposed to control *Dusp6*^{+/+}; *Apc*^{Min/+} mice (Figures 5b,c). In addition, expression of the proliferation marker PCNA was more elevated in double mutant *Dusp6*^{-/-}; *Apc*^{Min/+} polyps (Figures 5d,e). We have also determined phospho-ERK1/2 expression to assess MAPK signaling. As shown in Figure 5d, polyps from *Apc*^{Min/+} mice displayed consistently much higher phosphorylation levels of ERK1/2 in comparison to the corresponding benign epithelium (margin). This is consistent with the observation that ERK signaling activation drives intestinal tumorigenesis in *Apc*^{Min/+} mice (S. H. Lee et al., 2010). However, upon deletion of *Dusp6* in *Apc*^{Min/+} mice, phospho-ERK levels were barely increased in the intestinal margins of some *Dusp6* KO mice (lanes 7 and 9 vs. lanes 1, 3, and 5) but not in polyps (lanes 8, 10, and 12 vs. 2, 4, and 6). Taken together, these results suggest that *Dusp6* deficiency promotes intestinal tumor initiation and proliferation, in part by promoting ERK1/2 activation.

3.5 | Decreased DUSP6 expression is associated with human CRC progression

To determine DUSP6 contribution in human CRC cells, we reduced DUSP6 protein expression in established human CRC HT29 and HCT116 cell lines, by a specific anti-DUSP6 short hairpin RNA. DUSP6 suppression led to increased ERK1/2

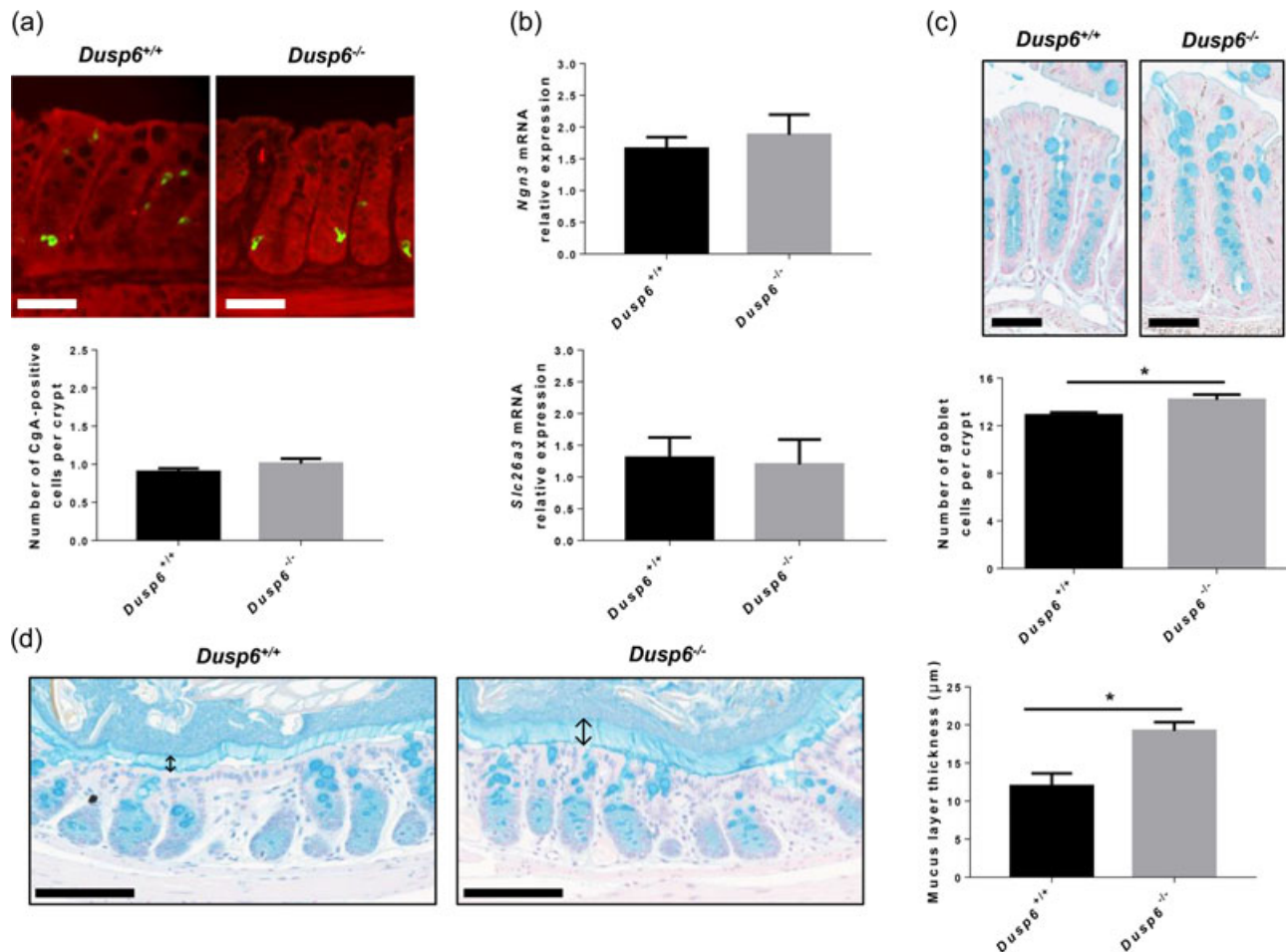


FIGURE 3 *Dusp6* deletion induces goblet cell expansion without affecting enteroendocrine and absorptive cell differentiation. (a) Chromogranin A immunofluorescence with Evan's blue counterstaining was performed on 12-week-old *Dusp6*^{+/+} and *Dusp6*^{-/-} murine colon sections to evaluate the number of enteroendocrine cells per crypt. Scale bars=50 μm (*n* = 7). (b) Relative *Ngn3* and *Slc26a3* mRNA expression was quantified by qPCR with RNAs isolated from 12-week-old murine total colonic extracts (*n* = 7). Relative expression was normalized to housekeeping genes *Pum1*, *Tbp*, and *Psmc4* expression. (c) Alcian blue staining was used to visualize Goblet cells in *Dusp6*^{+/+} and *Dusp6*^{-/-} colon sections. Scale bars: 50 μm. The number of Alcian blue positive cells per crypt was counted (*n* = 7). (d) Carnoy's fixation followed by Alcian blue staining was performed on 12-week-old *Dusp6*^{+/+} and *Dusp6*^{-/-} mouse distal colon sections to measure the mucus layer thickness (*n* ≥ 3). ↓Mucus layer. Scale bars=100 μm. Data are expressed as mean ± SEM. Student's *t* test; **p* ≤ 0.05. DUSP6: dual-specificity phosphatase 6; mRNA: messenger RNA; qPCR: quantitative polymerase chain reaction

phosphorylation levels in HT29 cells (Figure 6a). As expected, no difference was seen in the levels of phosphorylated MEK1/2. This increased ERK1/2 activity in DUSP6-depleted HT29 cells was associated with an enhanced capacity to grow in soft agar and to invade Matrigel (Figures 6b,c). In contrast, DUSP6-depleted HCT116 cells did not display increased MEK1/2 nor ERK1/2 activation or enhanced soft agar growth and invasion (Figures 6b, c). We also determined *DUSP6* mRNA expression levels in 66 human paired CRC specimens consisting of resection margins and primary tumors from our biobank. As shown in Figure 6d, relative amounts of *DUSP6* transcripts were significantly reduced in CRC tumors compared to normal specimens, mostly in advanced stage tumor samples (Figure 6e). Of note, decreased *DUSP6* gene expression was more prominent in microsatellite stable (MSS) tumors (Figure 6f). By contrast, there did not appear to be a significant difference relative to *KRAS* or *BRAF* mutations

(Figure 6g). These data suggest that DUSP6 inactivation may favor colorectal oncogenesis.

4 | DISCUSSION

We have shown here an important role of DUSP6, a cytoplasmic phosphatase that negatively regulates ERK1/2, in the maintenance of homeostasis in the colon. Indeed, *Dusp6* gene deletion in mice is sufficient to consistently increase, albeit modestly, endogenous ERK1/2 phosphorylation in the colonic mucosa. This increased ERK1/2 activity correlates with a stimulation of colonic crypt cell proliferation in *Dusp6* KO mice. By culturing colonic organoids, we further show that an increase in crypt cell proliferation in *Dusp6*^{-/-} mice is intrinsic to epithelial cells. These findings are in agreement with previous studies demonstrating the central role of the KRas/

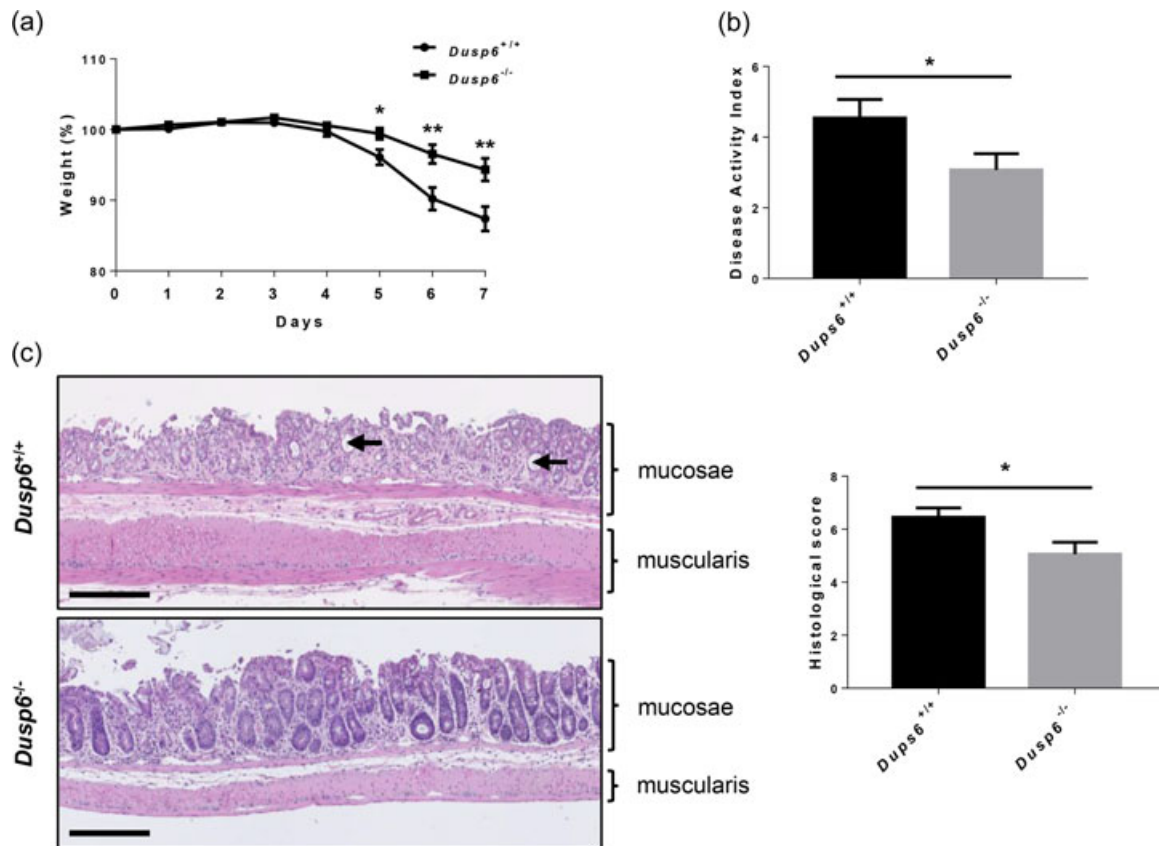


FIGURE 4 *Dusp6* deletion protects against DSS-induced colitis in mice. Eleven-week-old *Dusp6*^{+/+} and *Dusp6*^{-/-} mice were treated with 2% DSS in drinking water for 7 days. (a) Body weight was measured every day during treatment ($n = 21$). (b) Disease activity index of *Dusp6* knockout mice and control littermates was calculated by scoring stool softness, occult fecal blood, rectal bleeding, and colon rigidity at sacrifice ($n \geq 21$). (c) Hematoxylin and eosin staining was performed on *Dusp6*^{+/+} and *Dusp6*^{-/-} colon tissue to score the damage according to the extent of destruction of normal mucosal architecture (0, normal; 1, 2, and 3, respectively, mild, moderate, and extensive damage), presence and degree of cellular infiltration (0, normal; 1, 2, and 3, mild, moderate, and transmural infiltration, respectively), extent of muscle thickening (0, normal; 1, 2, and 3, respectively mild, moderate, and extensive thickening), presence or absence of crypt abscesses (0, absent; 1, present; see arrowheads) and the presence or absence of goblet cell depletion (0, absent; 1, present). ($n = 18$). Scale bars=250 μm . Data are expressed as mean \pm SEM. Student's t test; * $p \leq 0.05$, ** $p \leq 0.01$. DUSP6: dual-specificity phosphatase 6; DSS: dextran sulfate sodium

ERK MAPK pathway in the control of epithelial proliferation in both the small and large intestines (Aliaga et al., 1999; Feng et al., 2011; Haigis et al., 2008; Paquin et al., 2013; Rivard et al., 1999; Voisin et al., 2008). Notably, phosphorylated and activated forms of ERK1/2 have been mostly found in undifferentiated proliferative crypt cells in human intestine (Aliaga et al., 1999), hence supporting their function in epithelial cell proliferation. Interestingly, the expression of stem cell markers *Ascl2* and *Lgr5* is not affected in *Dusp6* KO colons, suggesting that crypt-based columnar stem cells were not affected by the *Dusp6* deficiency. Therefore, the increase number of proliferative cells observed in *Dusp6*^{-/-} mice may mostly result from an increased number of transit-amplifying cells, cells which are undifferentiated and in transition between stem cells and differentiated cells. These results are reminiscent of those of Feng et al. (2011) showing that KRas activation in the colonic epithelium while stimulating proliferation, does not expand the crypt stem cell compartment.

ERK signaling has been previously shown to negatively regulate enterocyte and enteroendocrine cell differentiation in the intestine

(Basak et al., 2017; Ding et al., 2001; Lemieux et al., 2011). However, our data show that the absence of *Dusp6* expression does not affect absorptive nor enteroendocrine cell differentiation in the colon. This might be explained by the modest increase of endogenous ERK1/2 activity observed in *Dusp6*^{-/-} colonic epithelium. Such modulation of ERK activity might not be sufficient to significantly perturb absorptive and endocrine cell differentiation pathways. However, *Dusp6* deletion is sufficient to deregulate colonic goblet cell number and function, suggesting that goblet cell differentiation is positively affected by ERK signaling and may be more sensitive to ERK1/2 activity changes. Accordingly, Feng et al. (2011) as well as Yamashita et al. (2014) previously observed goblet cell expansion in mouse intestines expressing oncogenic KRas or MEK1. Furthermore, activation of ERK signaling in airway epithelial and CRC cells promotes *MUC2* gene transcription and mucus production (Dilly et al., 2015; Hatayama, Iwashita, Kuwajima, & Abe, 2007; Kim et al., 2002; Kuracha, Thomas, Loggie, & Govindarajan, 2017; C. S. Lee, Perreault, et al., 2002; H.-W. Lee, Ahn, et al., 2002; Li et al., 1998; Perrais, Pigny, Copin, Aubert, &

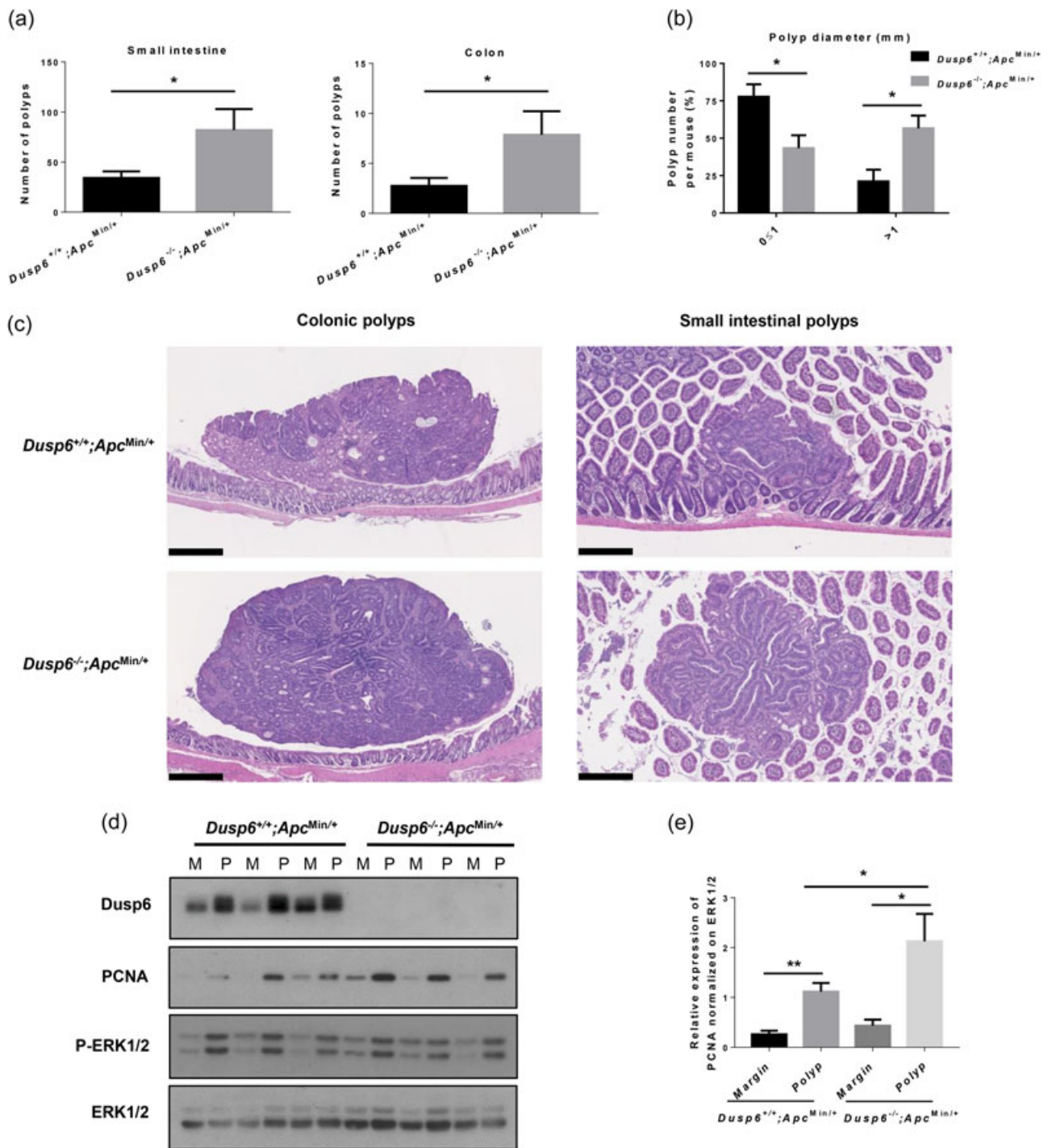


FIGURE 5 *Dusp6* deletion increases intestinal tumorigenesis in *Apc^{Min/+}* mice. (a) Polyp numbers were counted in 13-week-old *Dusp6^{+/+}; Apc^{Min/+}* and *Dusp6^{-/-}; Apc^{Min/+}* small intestine and colon ($n \geq 7$). (b) Small intestinal polyp size (diameter) was compared between *Dusp6^{+/+}; Apc^{Min/+}* and *Dusp6^{-/-}; Apc^{Min/+}* mice ($n = 5$). (c) *Dusp6^{+/+}; Apc^{Min/+}* and *Dusp6^{-/-}; Apc^{Min/+}* polyp histology was visualized with hematoxylin and eosin staining in the small intestine (scale bars: 250 μ m) and colon (scale bars: 500 μ m; representative of $n \geq 4$). (d) *Dusp6*, PCNA, and phosphorylated ERK1/2 levels were analyzed by western blot of polyp (P) and healthy margin (M) protein extracts ($n = 3$). ERK1/2 expression was used as loading control. Representative immunoblots are shown. (e) Densitometric analysis of PCNA expression relative to ERK1/2 ($n = 7$). Data are expressed as mean \pm SEM. Student's *t* test; * $p < 0.05$, ** $p < 0.01$. DUSP6: dual-specificity phosphatase 6; ERK: extracellular signal-regulated kinase; PCNA: proliferating cell nuclear antigen

Van Seuning, 2002; Suzuki, Takeuchi, Ishinaga, Basbaum, & Majima, 2008). Overall, these observations suggest a role for the Ras/ERK pathway in goblet cell fate and differentiation. However, the exact molecular mechanisms involved remain to be elucidated.

These modulations in crypt cell proliferation and differentiation observed in *Dusp6* KO intestine prompted us to determine whether *Dusp6* play a role in the intestinal response against injury. We, therefore, investigate a model of ulcerative colitis in the large

intestine. Treatment of wild-type mice with DSS induces acute colitis. Interestingly, *Dusp6* KO mice exhibited less weight loss, diarrhea, and bloody stools after DSS treatment as well as milder mucosal alterations at the microscopic level. Thus, *Dusp6* deficiency in IEC likely protects the colonic mucosae against injury-induced inflammation. Increased IEC proliferation, enhanced goblet cell numbers as well as mucus production may be all implicated in colitis protection.

Indeed, proliferation is important for wound healing after epithelial damage in the intestine and the mucus layer is protective against adhesion and invasion by microbes and antigens (Sturm & Dignass, 2008). For instance, mice knockout for *Muc2* spontaneously develops chronic inflammation in the colon and rectum (Van der Sluis et al., 2006). Additionally, Ruan et al. (2016) have recently measured the in vivo gut permeability in *Dusp6*^{-/-} mice using

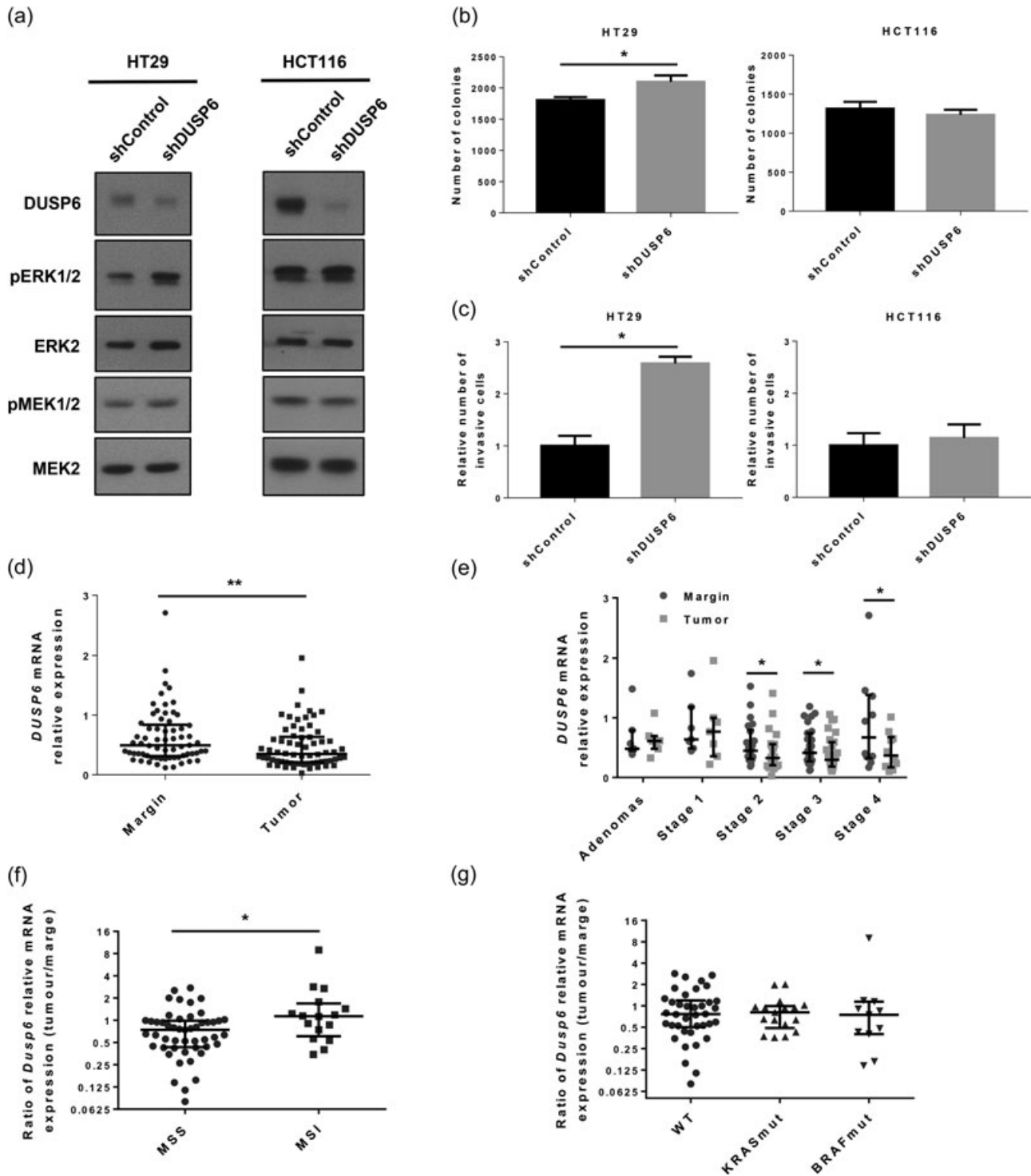


FIGURE 6 Continued.

fluorescein isothiocyanate dextran molecule and found reduced paracellular permeability in comparison to wild-type mice. Therefore, a tightening of the epithelial barrier could also contribute to the protective effect against colitis observed in *Dusp6*^{-/-} mice. Together, these data indicate that *Dusp6* maintains epithelial barrier function in colonic mucosae.

By contrast, another study (Bertin et al., 2015) showed that *Dusp6* deletion accelerated and exacerbated inflammation in interleukin-10 deficient (*Il10*^{-/-}) mouse model of colitis. The discrepancy between our data and the study of Bertin et al. may be explained by the different experimental models used for colitis induction. Herein, we used DSS which triggers inflammation by causing chemical injury directly to the intestinal epithelium, resulting in exposure of the lamina propria and submucosa to luminal antigens and bacteria (Low, Nguyen, & Mizoguchi, 2013). Thus, by stimulating goblet cell function as well as crypt proliferation and regeneration, *Dusp6* deletion may protect the colonic epithelium against erosion induced by DSS. By contrast, the enterocolitis in *Il10*^{-/-} mice is largely attributed to dysfunctional CD4⁺ T-cell activation (Roers et al., 2004). In their study, Bertin et al. (2015) clearly demonstrated that *Dusp6*-deficient CD4⁺ T cells have increased ERK1/2 activation and production of interferon γ upon TCR stimulation. They also showed that *Dusp6*^{-/-} naïve CD4⁺ T cells exhibited a greater propensity to differentiate along the Th1 axis in vitro. Therefore, it is not surprising that *Dusp6* deletion exacerbated spontaneous colitis in the *Il10* deficient mice.

Aberrant activation of ERK signaling is commonly linked to intestinal tumorigenesis and CRC. Notably, mutations in genes encoding *KRAS* and *BRAF*, the upstream activators of ERK1/2 kinases, occur in approximately 40% and 10%, respectively, of all CRCs (Andreyev, Norman, Clarke, Cunningham, & Oates, 1998; Davies et al., 2002; Rajagopalan et al., 2002; Samowitz et al., 2000). Furthermore, several studies have demonstrated that inhibition of MEK/ERK signaling prevents CRC cell growth in cell culture and mouse xenografts (Balmanno et al., 2009; Kress, Raabe, & Feller,

2010; Lemieux et al., 2009; Sebolt-Leopold et al., 1999; Solit et al., 2006; Voisin et al., 2008; Wang et al., 2013). However, *DUSP* functions in the oncogenicity of *KRAS*/ERK pathway in CRC are not known. Herein, we demonstrated that *Dusp6* deletion promotes intestinal tumor load and size in *Apc*^{Min/+} mice, supporting the requirement for ERK1/2 activity in *Apc*^{Min/+}-dependent murine intestinal tumorigenesis (S. H. Lee et al., 2010). Indeed, polyps from *Apc*^{Min/+} mice displayed much higher phosphorylation levels of ERK1/2 in comparison to the corresponding benign epithelium (margin). Surprisingly, we did not detect a further increase in phospho-ERK levels in *Dusp6*-deficient polyps. The reason why this was not observed in polyps is not clear but is consistent with other reports (Maillet et al., 2008). It is possible that ERK1/2 phosphorylation is induced earlier during the tumorigenic process of *Apc*^{Min/+} mice in the absence of *Dusp6*. Herein, the mice were killed at 3 months of age to obtain a significant number of polyps (Moser et al., 1990). To better analyze the regulation of ERK activity during polyposis, it would have been necessary to also sacrifice mice earlier, at the age of 1 or 2 months. That being said, the possibility that *Dusp6* dephosphorylates other targets than ERK1/2 cannot be totally excluded. For instance, *Dusp6* has been shown to interact with other kinases such as CK2 (Hagan, Knutson, & Lange, 2013) and ERK5 (Razmara, Eger, Rorsman, Heldin, & Lennartsson, 2012; Sarközi et al., 2007). In this regard, ERK5 is particularly interesting since this MAPK can rescue intestinal epithelial turnover and tumor cell proliferation upon ERK1/2 inhibition (de Jong et al., 2016).

Notably, we found that *DUSP6* gene expression decreases in patient-derived colorectal tumors, mostly in advanced stage tumors. While we did not detect a significant association between *DUSP6* expression and *KRAS* or *BRAF* mutations in the small number of adenocarcinomas analyzed, a correlation between low *DUSP6* transcript levels and MSS status of tumors was observed. Interestingly, MSS tumors are generally more aggressive than MSI tumors and are more often associated with lymph-node metastases and distant spread

FIGURE 6 Decreased *DUSP6* expression is associated with human colorectal cancer progression. HT29 and HCT116 colorectal carcinoma cells were stably infected with lentiviruses encoding for a control shRNA or a shRNA against *DUSP6*. (a) ERK phosphorylation, MEK phosphorylation, and *DUSP6* expression were analyzed by western blot. ERK2 and MEK2 expression were used as loading control. Representative immunoblots are shown. (b) HT29 and HCT116 cells were cultured in soft agarose for 14 days before MTT staining. Graphs are representative of three independent experiments. Data are expressed as mean \pm SEM. Student's *t* test; **p* \leq 0.05. (c) Invasion of shControl- and sh*DUSP6*-expressing HT29 and HCT116 cells was evaluated in Matrigel-coated transwells. After 72 hr for HT29 cells and 48 hr for HCT116 cells, invading cells were fixed, stained with 1% crystal violet and counted (*n* = 3 independent experiments for HT29 cells and *n* = 5 for HCT116 cells). Data are expressed as mean \pm SEM. Student's *t* test; **p* \leq 0.05. (d) Relative *DUSP6* mRNA expression was analyzed by qPCR with RNAs isolated from human colorectal tumor specimens compared to paired adjacent healthy tissue. Relative expression was normalized to housekeeping genes *MRPL19*, *SDHA*, and *YWHAZ* expression (*n* = 66). Data are expressed as a median \pm interquartile range. The Wilcoxon signed-rank test; ****p* \leq 0.01 (e) Relative *DUSP6* mRNA expression was assessed by qPCR with RNAs isolated from colorectal tumors and paired healthy adjacent tissue separated by stage (*n* = 7 for adenomas and Stage 1, *n* = 22 for Stage 2, *n* = 27 for Stage 3 and *n* = 10 for Stage 4). Relative expression was normalized to housekeeping genes *MRPL19*, *SDHA*, and *YWHAZ* expression. Data are expressed as a median \pm interquartile range. The Wilcoxon signed-rank test; **p* \leq 0.05. (f) Ratio of *DUSP6* mRNA expression (tumor/paired adjacent healthy tissue) was compared between MSS (*n* = 50) and MSI (*n* = 16) patients. Relative expression was normalized to housekeeping genes *MRPL19*, *SDHA*, and *YWHAZ* expression. Data are expressed as a median \pm interquartile range. Mann-Whitney *U* test; **p* \leq 0.05. (g) Ratio of *DUSP6* mRNA expression (colorectal tumor/paired adjacent healthy tissue) was compared between wild-type (*n* = 38), *KRAS*-mutated (*n* = 17), and *BRAF*-mutated (*n* = 11) patients. Relative expression was normalized to housekeeping genes *MRPL19*, *SDHA*, and *YWHAZ* expression. Data are expressed as a median \pm interquartile range. Mann-Whitney *U* test. *DUSP6*: dual-specificity phosphatase 6; ERK: extracellular signal-regulated kinase; MEK: mitogen-activated protein kinase/Erk kinase; mRNA: messenger RNA; qPCR: quantitative polymerase chain reaction; shRNA: short hairpin RNA

(Popat, Hubner, & Houlston, 2005). Therefore, one could speculate that the low DUSP6 expression in MSS tumor cells would contribute to their invasive and metastatic properties by enhancing ERK activity. Accordingly, in HT29 human colon carcinoma cells, silencing of DUSP6 expression resulted in enhanced activation of ERK1/2 and this correlates with increased anchorage-independent growth ability and invasion capacity. Together, these findings provide evidence that DUSP6 expression limits intestinal tumor growth and progression.

Surprisingly, DUSP6 silencing did not alter the oncogenic properties of HCT116 cells nor ERK activation levels. The fact that HT29 cells are *BRAF* mutated while HCT116 cells bear a *KRAS* mutation (Davies et al., 2002) is a plausible explanation for this discrepancy between these two cell lines. Indeed, there is a correlation between ERK1/2 activation and *BRAF*, but not *KRAS*, mutation status in CRC cells and tumors (Yeh et al., 2009). In addition, we cannot exclude that HCT116 cells may express higher levels of other DUSP, which in turn can also negatively regulate ERK phosphorylation. For instance, we previously observed higher DUSP4 expression levels in HCT116 cells in comparison to HT29 cells (Cagnol & Rivard, 2013). Regardless of the reason that explains this difference in ERK phosphorylation modulation between the two cell lines, our results suggest that DUSP6 silencing must result in increased ERK1/2 phosphorylation to stimulate anchorage-independent growth ability and invasion capacity in CRC cells.

A tumor suppressive role for DUSP6 has also been suggested in several cancers (Chan et al., 2008; Furukawa, Sunamura, Motoi, Matsuno, & Horii, 2003; Okudela et al., 2009; Xu, Furukawa, Kanai, Sunamura, & Horii, 2005). Accordingly, expression levels of DUSP6 are indeed reduced in pancreatic, ovarian and lung cancers (Chan et al., 2008; Furukawa et al., 2003; Okudela et al., 2009; Xu et al., 2005) and DUSP6 overexpression suppresses cancer cell growth (Chan et al., 2008; Furukawa et al., 2003; Okudela et al., 2009). However, other studies have hinted at an oncogenic function for DUSP6. Indeed, DUSP6 is upregulated in various cancer cell lines including those from glioblastomas, papillary thyroid carcinoma and gastric cancers and its enforced expression in these cancer cell lines clearly increases their growth, migration and/or tumorigenic potential in vivo (Degl'Innocenti et al., 2013; Messina et al., 2011; Wu et al., 2018). The mechanisms explaining why DUSP6 can act as a tumor suppressor in some cancers and as an oncogene in others still remain to be determined.

In conclusion, our results demonstrate that the DUSP6 phosphatase, by limiting the activation of ERK signaling, regulates colonic inflammatory response and protects the colonic epithelium against oncogenic stress.

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AUTHOR'S CONTRIBUTIONS

K. B., M.-J. L., A. M., and S. C. performed research. J. C. C. provided the tumor specimens. N. R., K. B., M.-J. L. and S. C. analyzed data. M.-J. L., N. R., and K. B. wrote the paper. N. R. designed research. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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