mTORC1 Prevents Epithelial Damage During Inflammation and Inhibits Colitis-Associated Colorectal Cancer Development

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Abstract

Epithelial cells lining the intestinal mucosa constitute a selective-semipermeable barrier acting as first line of defense in the organism. The number of those cells remains constant during physiological conditions, but disruption of epithelial cell homeostasis has been observed in several pathologies. During colitis, epithelial cell proliferation decreases and cell death augments. The mechanism responsible for these changes remains unknown. Here, we show that the pro-inflammatory cytokine IFNγ contributes to the inhibition of epithelial cell proliferation in intestinal epithelial cells (IECs) by inducing the activation of mTORC1. Activation of mTORC1 in response to IFNγ was detected in IECs present along the crypt axis and in colonic macrophages. mTORC1 inhibition enhances cell proliferation, increases DNA damage in IEC. In macrophages, mTORC1 inhibition strongly reduces the expression of pro-inflammatory markers. As a consequence, mTORC1 inhibition exacerbated disease activity, increased mucosal damage, enhanced ulceration, augmented cell infiltration, decreased survival and stimulated tumor formation in a model of colorectal cancer CRC associated to colitis. Thus, our findings suggest that mTORC1 signaling downstream of IFNγ prevents epithelial DNA damage and cancer development during colitis.

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Introduction

Intestinal epithelial cells (IEC) form a selective barrier that separates luminal contents from the underlying tissue, thus maintaining a functional barrier requires the number of IEC to remain constant. Therefore, a perfect balance between proliferating and dying cells is essential for maintaining epithelial homeostasis and proper function of the intestinal epithelium. Disruption of IEC homeostasis actively contributes to development and establishment of several pathologies, including inflammatory bowel diseases (IBD) and colorectal cancer [1].

During colitis, numerous signaling pathways linked to the development and progression of colorectal cancer have been also associated with the inhibition of cell proliferation [2–4]. Therefore, understanding the specific mechanism controlling proliferation and IEC death in the colon is an important step in the identification of the machinery implicated in the development of several colonic pathologies.

Signaling networks, downstream of several molecules including cytokine receptors, provide a unique mechanism to integrate internal and external stimuli aimed to coordinate cellular responses. Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase that regulates several biological processes including proliferation, survival and growth and is activated by cytokine receptors [5]. Its major function is mediated by the activation of the protein kinase B, also known as Akt [6]. Akt in turns activates several other effectors including the protein kinase complex mTORC1, a key regulator for metabolism and cell growth. mTORC1 is constituted by mTOR, Raptor, and mLST8, and the inhibitors of the complex PRAS40 and DEPTOR. mTORC1 main effects are controlled through rapamycin-sensitive phosphorylation of its substrates, S6 kinase 1 (S6 K1) and eIF4E-binding protein 1 (4E-BP1). mTORC1 activation is also important for inhibiting autophagy and increasing of protein and lipid synthesis, thus mTORC1 is necessary for preventing futile cycles of synthesis and degradation of cellular components [7,8]. mTORC1 has been implicated in the development of several pathological processes in the gut, including colorectal cancer development [9] and colitis, however the mechanism underlying those processes remain poorly understood.

Here, using *in vivo* and *in vitro* models of inflammation we demonstrated that IFNγ, a pro-inflammatory Th1cytokine that is upregulated in the colonic mucosa of colitic patients [10–12], negatively regulates IEC proliferation by inducing inactivation of Wnt/β-catenin signaling through mTORC1 [2,13–15]. Here we sought to analyze the role of mTORC1, a downstream target of Akt in the regulation of this process. We demonstrated that IFNγ induces activation of mTORC1 in IECs located along the crypt axis but also in immune cells (*e.g.* macrophages) present at the colonic mucosa. Interestingly, we observed that pharmacological inhibition of mTORC1 increased the number of proliferating IECs in the mucosa of colitic mice but this process resulted in enhanced epithelial damage and ulceration at the epithelium. Thus, mTORC1 inactivation augmented disease activity and mortality in a colitis model induced by the administration of DSS. Furthermore, mTORC1 inhibition triggered β-catenin dependent DNA damage in IECs and therefore stimulates CRC development associated to colitis. Such effects occurred because after mTORC1 inhibition macrophages failed to polarize to M1-phenotype and therefore an anti-inflammatory environment is created in the colon. Thus our findings strongly suggest that inhibition of mTORC1 during colitis could play important role in the development of colorectal cancer. However, they also demonstrated in the colonic mucosa the activation of mTORC1 by IFNγ plays a protective role not only in the maintenance of the epithelial integrity but also in the induction of IEC DNA damage.

Materials and Methods

Animals, Cell Culture and Crypt Isolation

Six- to eight-week-old (22–25 g of weight) C57BL/6 J or Lgr5-EGFP-IRES-creERT2 (β-catenin reporter mice; The Jackson Laboratories, Bar Harbor, ME) male mice were kept in standard day/night cycles conditions with free access to food and water. All experiments were approved by the Internal Committee for Care and Use of Laboratory Animals (CICUAL). The human IEC lines SW480 and RKO were obtained from ATCC (Manassas, VA) and cultured according to the provided protocols. Crypt isolations were performed as previously reported [14].

Antibodies and Reagents

2Antibodies against GAPDH sc-322 (Santa Cruz Biotechnology, Santa Cruz, CA), pSTAT1 Tyr701 #9167, pS6 #15967, pAkt Ser473 #4060, Akt1 #2967, pGSK3β #5558, pH2AX (γ-H2AX) mAb #9718, pp53 #9286, CHK1 # 2348 and pBRCA1 #9009 were obtained from Cell Signaling Technology (Danvers, MA). F4/80 Monoclonal Antibody (BM8) coupled to Biotin (eBioscience, Waltham, MA) was used according to manufacturer's instructions. p-Histone H3, ab32107 was obtained from Abcam (Cambridge, MA). Alexa conjugated antibodies were obtained from Thermo Fisher Scientific (Waltham, MA). AffiniPure goat and rabbit anti-horseradish peroxidase (HRP) were obtained from Jackson Immunoresearch (San Diego, CA) and 4′,6-Diamidino-2-phenylindole (DAPI) SC-3598 from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant mouse IFNγ (Pepro-Tech, Rocky Hill, NJ) was dissolved in 0.002% mouse serum albumin (MSA; Sigma-Aldrich, St. Louis, MO) and used at 2.5 μg/kg of weight. LiCl (Sigma-Aldrich, St Louis, MO) was used at 4 mg/mice and 4 μM *in vitro*. Rapamycin used as previously reported by us [14] Doxorubicin was obtained from Pfizer and used at 3.45 μM. AZD8055 and XAV939 were used as previously reported by us [16,17]. Dextran sulfate sodium YD318041799 (Carbosynth, San Diego, CA) was dissolved at 3% in drinking water.

Cytokine Treatment

Mice were randomly divided into groups. IFNγ was dissolved in PBS/MSA (0.02%) and administered intraperitoneally; control mice were injected with carrier alone. Two hours after cytokine administration mice were euthanized and colons processed for immunofluorescence and Western blot.

In Vivo Administration of Inhibitors and DSS-Induced Colitis Model

LiCl (4 mg/mice) was dissolved in PBS and Rapamycin (2 mg/kg) and AZD8055 (7.5 mg/kg) were dissolved in DMSO. The inhibitors were injected last 3 days of DSS treatment. Control group received vehicle alone. DSS-induced colitis model was previously described [18]. After treatment mice were euthanized and colons processed for immunofluorescence and Western blot analysis.

Proliferation Analysis and TOP-FOP Reporter Activity

Proliferation was assessed *in vivo* by intraperitoneal injection of 100 μg 5-ethynyl-2-deoxyuridine (EdU, Invitrogen). EdU incorporation was determined with a Click-iT EdU Cell Proliferation Kit (Invitrogen), according to the supplier's instructions. β-Catenin TCF/LEF reporter expression and *in vitro* cell proliferation assay. TCF reporter construct activity was measured using Dual Luciferase Reporter system (Promega, Madison WI) according to the manufacturer's instructions.

Immunofluorescence, Histology and Western Blot

Immunofluorescence, immunohistochemistry and Western blot of colonic mucosa were performed as previously described by us [14].

Statistical Analysis

All statistical analysis were performed in Prism 6.0 (GraphPad Software). Shapiro–Wilk, one-way ANOVA, two-tailed T, Dunnett,

and Bonferroni tests were performed $P < .05$ was considered statistically significant.

Results

Colitis Induces Activation of mTORC1 in Colonic Mucosal Cells

Epithelial barrier destruction elicited by the Th1 pro-inflammatory cytokine, IFNγ, has been linked to uncontrolled activation of several signaling pathways, including PI3K [13]. Thus, we used an *in vivo* model of intestinal inflammation to investigate the activation of PI3K signaling during colitis. As shown in Figure 1A, after 4 days of colitis induction lower incorporation of EdU indicating decreased proliferation was noticed in the mucosa of colitic mice. Furthermore, the presence of high levels of pAkt473 (pAkt), β-catenin serine 552 (pβcat$_{552}$) and pS6 in the mucosa of colitic mice demonstrated that inflammation enhances the activation of Akt/mTORC1 signaling (Figure 1B).

Akt activation increases in epithelial cells during colitis [2], thus we next analyzed the status of mTORC1 in those cells. To this end, pS6 presence was examined in colonic crypts isolated from control and colitic mice. As shown in Figure 1C, phosphorylation of pS6 significantly increased in colonic IECs of DSS treated mice when compared with control animals. Phosphorylation of S6 in the mucosa
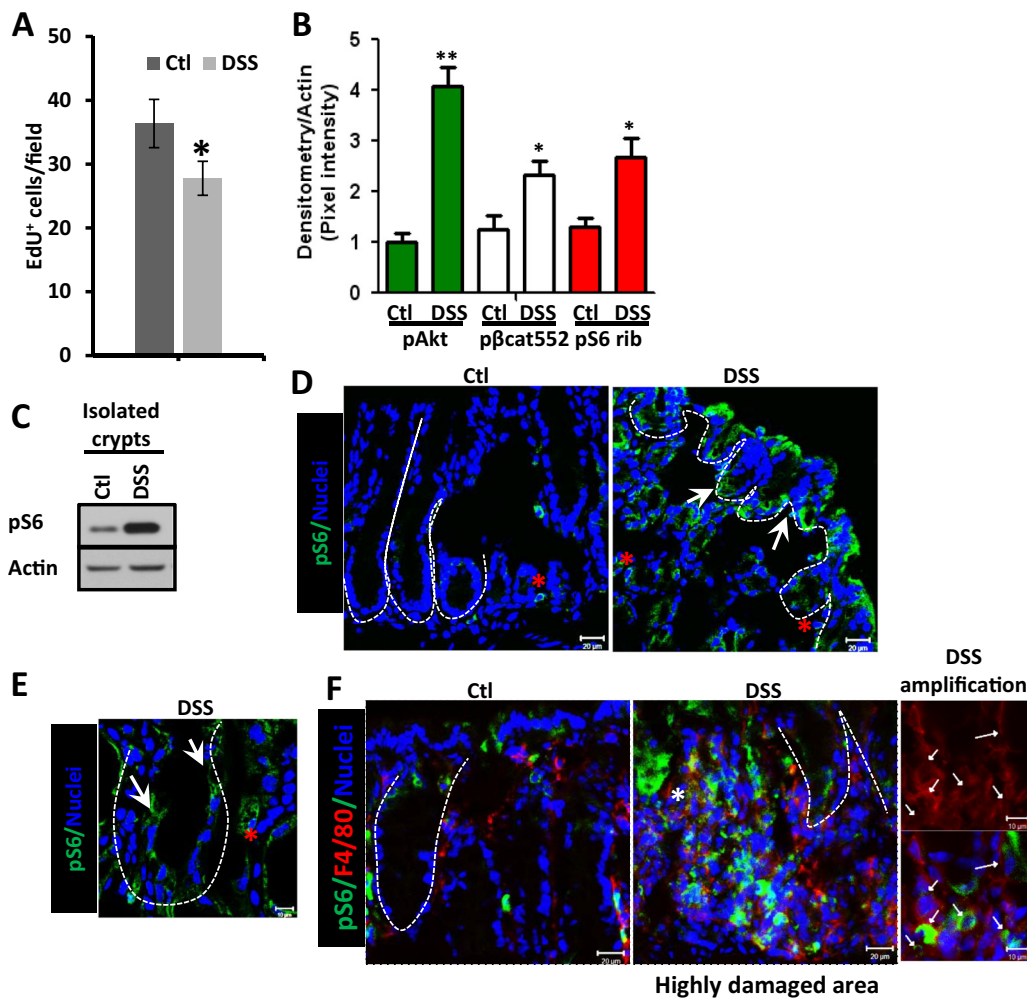

Figure 1. mTORC1 is activated in colonic IECs and macrophages during colitis. A. EdU (5-ethynyl-2′-deoxyuridine) incorporation in the colonic mucosa of colitic mice was evaluated by immunofluorescence. Four days DSS treated mice were injected intraperitoneally with EdU (20 mg/kg) and 2 h later euthanized. Quantification of EdU incorporation was analyzed in 100 crypts in each animal. Bar graphs represent the number of cells EdU-positive per field. Data are expressed as mean ± SEM. n = 3. *$P < .05$. B. Densitometric analysis of pAkt, pβ-catenin552 and pS6 in the mucosa of colitic mice was performed. DSS treatment was carried out for 4 days. Data are expressed as mean ± SEM. n = 3. *$P < .05$, **$P < .01$. C. pS6 was analyzed by Western-blotting colonic crypts of control and DSS treated animals. C57BL/6 J mice were treated with 3% DSS dissolved in tap water for 4 days. Actin was used as a loading control. n = 3. D. Localization of pS6 (green) in the colonic mucosa of C57BL/6 J mice treated with DSS for 4 days. Red asterisk = non-epithelial cells pS6-positive. Dashed line marks the crypt border. White arrow = Crypt base IECs pS6-positive. Nuclei = (Blue). Bar = 20 μm. n = 3. E. Immunolocalization of pS6 (green) in the base of the colonic crypt of C57BL/6 J mice treated with DSS for 4 days. Dashed line marks the crypt border. White arrow marks IECs positive for pS6 and red asterisk designates non-epithelial cells pS6-positive. Nuclei = (Blue). Bar = 10 μm. n = 6. F. pS6 (green) and F4/80 (Red) staining in the colonic mucosa of C57BL/6 J mice treated with DSS for 4 days. Dashed line marks the crypt border. After DSS treatment images were collected from areas with highly damaged epithelium that were bordering ulcerated areas. White asterisk designates double positive cells. Nuclei = (Blue). Bar = 20 μm. n = 3.

of colitic mice was detected in both IECs and non-epithelial cells in the colonic mucosa. IECs pS6-positive were located along the whole crypt axis including at the crypt base (Figure 1, D and E, arrows). Non-epithelial cells pS6-positive enriched in highly damaged areas and some of them were positive for the macrophage marker F4/80 (Figure 1F and Supplementary Figure 1A). Interestingly, non-epithelial cells pS6-positive were in close apposition of IECs and were noticed in all conditions (Figure 1, D–F, asterisks). Thus we conclude that during colitis mTORC1 is strongly activated in IECs lining the colonic crypt and in immune cells that are in close proximity to ulcerated areas.

IFNγ Induces Activation of mTORC1 in Colonic Mucosa Cells

IFNγ activates PI3K/Akt signaling in the mucosa of colitic mice and reduces proliferation [2,3,14,16], therefore we analyzed mTORC1 status in SW480 and RKO cells treated with IFNγ for 3 h. As shown in Figure 2A increased phosphorylation of STAT1 phosphorylation at Y701 was observed after IFNγ stimulation demonstrating the functionality of our assay. Furthermore, phosphorylation of Akt at ser473, β-catenin at ser552, mTOR at ser2448 and P70S6K at Thr389 was induced after cytokine treatment. Additionally increased phosphorylation of serine 235/236 in S6 was always noticed (Supplementary Figure 1B). No changes in total Akt, β-catenin, mTOR, P70S6K or S6 protein levels were noticed in any condition.

Next we analyzed the activation mTORC1 in the mucosa of C57BL/6 J animals that were administered intraperitoneally with IFNγ. As shown in Figure 2B the administration of IFNγ enhanced the phosphorylation of Akt, mTOR and S6. Furthermore, pS6

staining was almost absent the mucosa of control animals but a strong presence of the molecule was induced in IECs of C57BL/6 J stimulated with IFNγ (Figure 2, C and D, arrows). Interestingly, non-epithelial cells pS6-positive surrounding the crypt base were noticed in both, MSA and IFNγ treated mice (Figure 2C, red asterisks). Those cells expressed the macrophage marker F4/80 (Figure 2D, white asterisk). The number of F4/80[+]/pS6-positive cells increased after cytokine treatment (Figure 2, C and D, asterisks). Taken together these results strongly suggest that IFNγ induces activation of mTORC1 in IECs and macrophages at the colonic mucosa.

mTORC1 Reduces Proliferation in the Mucosa of Colitic Mice

IFNγ inhibits proliferation of IECs *in vitro* (Supplementary Figure 1B), thus we investigated the role of mTORC1 in this process. To this purpose, mTORC1 signaling was inhibited with LiCl, AZD8055 or Rapamycin in SW480 cells stimulated with IFNγ. Rapamycin inhibits mTORC1 directly and AZD8055 targets mTOR [8,18,19], but in contrast LiCl has a more broad specter that results in Akt inhibition in cells stimulated with cytokines [20]. As shown in Figure 3A, IFNγ treatment increased pGSK3β, pS6 and pAkt levels demonstrating activation of PI3K/mTORC1 signaling in SW480 cells. LiCl strongly enhanced pGSK3β presence but reduced pS6 and pAkt. AZD8055 treatment reduced pGSK3β, pS6 and pAkt levels and in contrast rapamycin inhibited S6 phosphorylation but failed to prevent Akt activation or GSK3β inhibition. No changes in total Akt, S6 and GSK3β protein levels were noticed after any treatment. Thus in conjunction this results strongly suggests that IFNγ activates mTORC1 downstream of PI3K/Akt in colonic IECs.
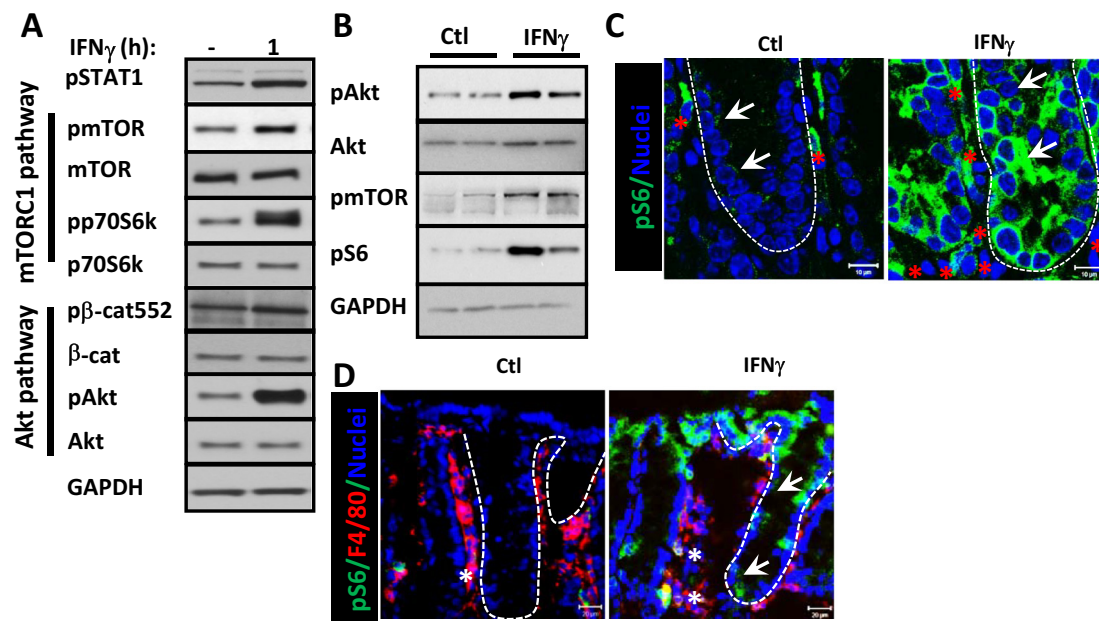

Figure 2. mTORC1 activation in the colonic mucosa is mediated by IFNγ. A. pAkt, pβ-catenin, pmTOR, pP70S6K and total proteins were analyzed in cell lysates of T84 cells treated with IFNγ. IFNγ (100 U/ml) treatment was carried out for 1 h. GAPDH was used as a loading control. n = 10. B. pmTOR, mTOR, pAkt, Akt, pS6 and S6 presence was assessed in colonic homogenates obtained from C57BL/6 J treated with MSA (Ctl) or IFNγ. IFNγ was administered intraperitoneally 2 h before animals were euthanized. GAPDH was used as a loading control. n = 3. C. Localization of pS6 (green) in the colonic mucosa of C57BL/6 J mice. IP injection of MSA (Ctl) or IFNγ was performed 2 h before animals were euthanized. Dashed line marks the crypt border. White arrow = IECs positive for pS6. Red asterisk = non-epithelial cells pS6-positive. Nuclei = (Blue). Bar = 10 μm. n = 6. D. pS6 (green) and F4/80 (Red) staining in the colonic mucosa of C57BL/6 J mice. Dashed line marks the crypt border. IP injection of MSA (Ctl) or IFNγ was performed 2 h before animals were euthanized. White arrow marks IECs positive for pS6 and white asterisk designates macrophages pS6-positive. Nuclei = (Blue). Bar = 20 μm. n = 3.
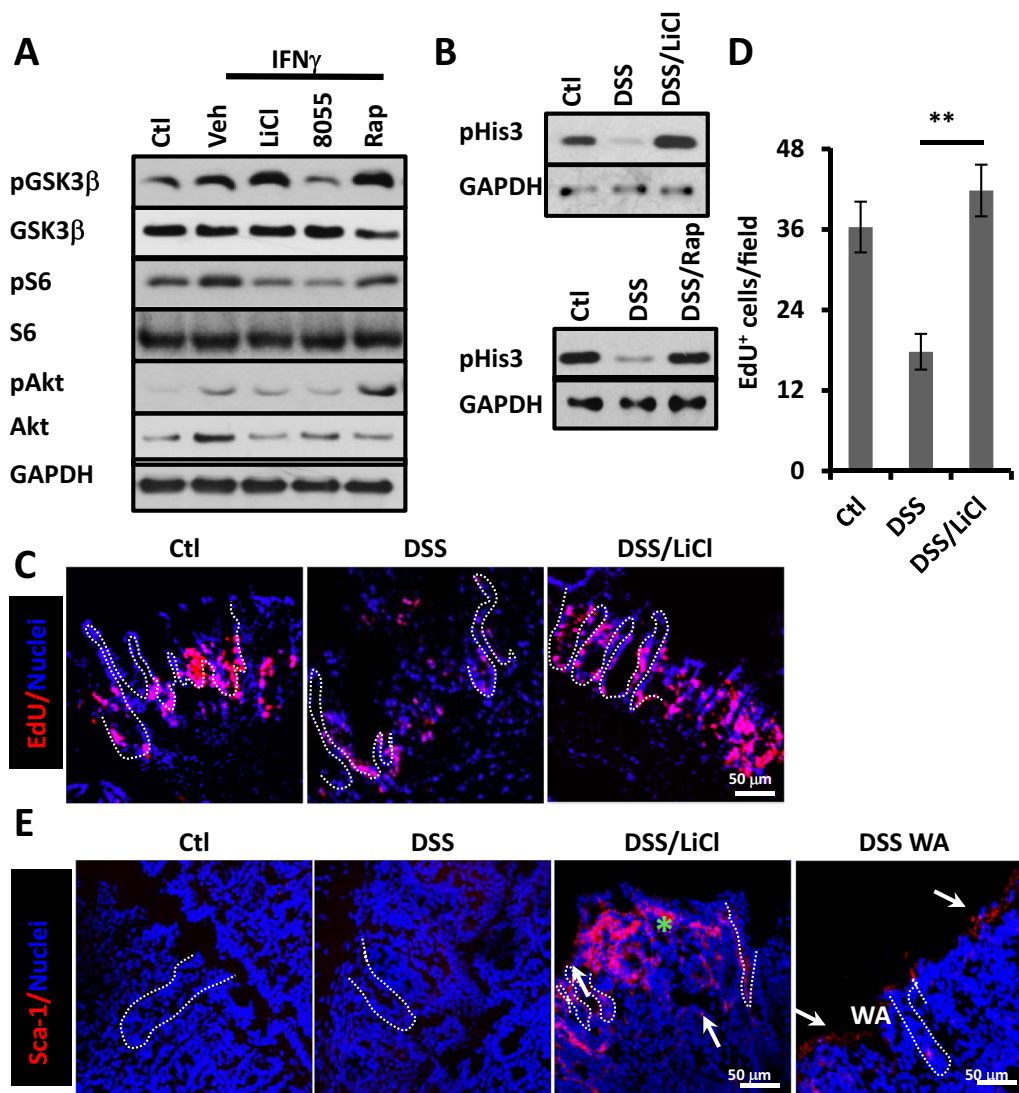
Figure 3. mTORC1 decreases proliferation in the colonic mucosa during inflammation. A. pAkt, Akt, pS6, S6, pGSK3β, and GSK3β were evaluated in cell lysates of SW480 exposed to IFNγ. LiCl, AZD8055 and Rapamycin were added 30 min before cytokine stimulation. GAPDH was used as a loading control. n = 3. B. pHist3 was evaluated in homogenized colonic tissue obtained from Ctl, DSS, DSS/LiCl and DSS/Rapamycin treated animals. DSS treatment was carried out for 5 days and the last 4 days animals received a daily IP injection of LiCl (4 mg; [49]) or Rapamycin (2 mg/Kg; [21]). GAPDH was used as a loading control. n = 4. C. Incorporation of EdU (Red) was analyzed in the mucosa of colitic mice treated with LiCl or carrier alone. DSS treatment was carried out for 5 days and the animals received a daily IP injection of LiCl for the last 3 days. Nuclei = blue. n = 3. Bar = 50 μm. n = 3. D. Quantification of EdU incorporation in DSS and DSS/LiCl treated animals. Bar graphs represent the number of cells EdU-positive per high power field. Data are expressed as mean ± SEM. n = 3. **P < .01. E. Localization of Sca-1 (red) in the colonic mucosa of C57BL/6 J mice treated with DSS or DSS/LiCl. DSS treatment was carried out for 5 days and the animals received a daily IP injection of LiCl for the last 3 days. White arrow = IECs positive for Sca-1. Green asterisk = non-epithelial cells Sca1-positive. WA = Wounded area. Nuclei = (Blue). Bar = 50 μm. n = 3.

Next we analyzed the effect of inhibiting mTORC1 in the regulation of IEC proliferation in the mucosa of DSS-treated mice. To this end C57BL/6 J mice that were induced to colitis for 5 days received a daily shot of LiCl or rapamycin since day three of treatment. As shown in Figure 3B colitis induction reduces pHist3 protein levels, however strong phosphorylation of the molecule was noticed in mucosal samples of colitic mice injected with LiCl or Rapamycin. Of note, contrary to the observed with LiCl, inhibition of Akt by Akt inhibitor VIII cannot reverse the reduction in cell proliferation induced by DSS treatment (Supplementary Figure 1C). Hypophosphorylation of pS6 and pAkt demonstrated the efficiency

of our treatments. No changes in total S6 and Akt were noticed with any treatment. In addition, intestinal proliferation analysis carried out by EdU incorporation corroborated that inhibition of mTORC1 by LiCl stimulated cell proliferation in IEC in the mucosa of colitic mice (Figure 3, C and D). Interestingly, immunofluorescence staining for the Stem Cell Antigen-1 (Sca-1), a marker expressed only in new epithelial cells in the repairing epithelium [21] was absent in IEC of the intact mucosa of control or DSS-treated mice but as expected was identified in IECs lining ulcerated areas in colitic animals (Figure 3E, WA). However, LiCl administration to colitic mice strongly enhanced the expression of Sca-1 in the mucosa. Sca-1 presence

was detected in both, IECs (white arrow) and non-epithelial cells (green asterisk) located through the whole mucosa and not only around ulcerated areas as observed with the DSS-only treated mice. Thus all these results strongly suggest that inhibiting mTORC1 signaling by LiCl or Rapamycin during colitis stimulates proliferation and generation of new cells in the colonic mucosa in a process that is independent of the integrity status of the epithelium.

mTORC1 Prevents Epithelial Cell Death During Colitis

Next we analyzed the effect of inhibiting mTORC1 in the development and progression of colitis. Thus, C57BL/6 J mice were treated with DSS alone or DSS plus LiCl. As shown in Figure 4A, LiCl injection enhanced weight loss during colitis and induced mortality in colitic mice starting at 6 days of treatment (Arrow). Furthermore, LiCl administration resulted in a high Disease Activity Index (DAI) when compared with animals treated with DSS alone (Figure 4B). The inhibition of mTORC1 by Rapamycin also enhanced colitis-like symptoms in DSS-treated mice, but the animals

survived the whole treatment (data not shown). However, the administration of Akt inhibitor VIII reduced colitis development after DSS-treatment as previously reported by us [2]. AZD8055, a more powerful and specific inhibitor for mTOR [18,19] displayed similar effects to the observed with LiCl, as shown by enhanced weight loss and increased mortality in colitic mice (Supplementary Figure 2A). Also, DAI in DSS/LiCl and DSS/AZD8055 treated mice was comparable and significantly higher than the observed in mice receiving DSS alone (Figure 4B). Colon length in DSS, DSS/LiCl or DSS/AZD8055 treated mice displayed no significant variations in size, demonstrating that the presence of the inhibitors does not affect this parameter (Figure 4C). However, macroscopic inspection revealed generalized tissue destruction in the colonic tissue obtained from deceased animals (Supplementary Figure 2B). Histological examination of hematoxylin–eosin stained sections shown epithelial lesions throughout the mucosa with visible alterations of epithelial structure in colitic mice (Figure 4D, asterisk). Ulcerations (arrowhead) and edema were clearly augmented in the mucosa of colitic
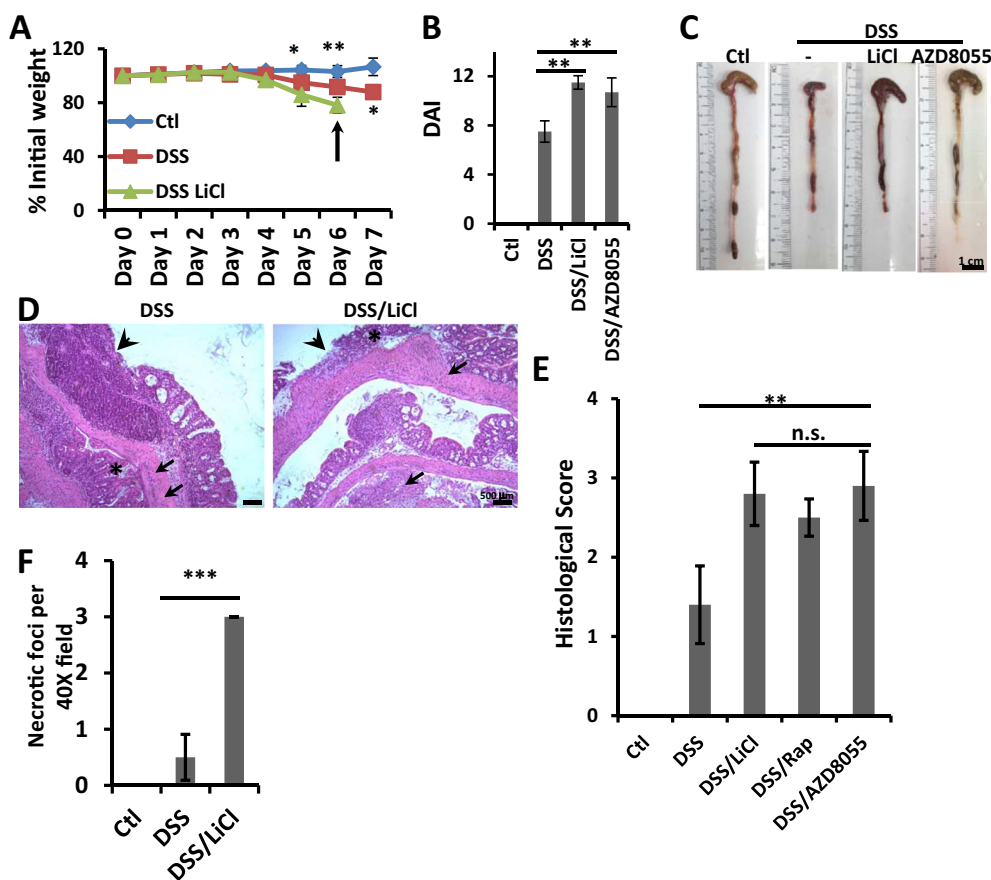

Figure 4. mTORC1 inhibition worsens epithelia damage during colitis. A. Percentage of body weight change for mice treated with tap water (Ctl), DSS or DSS plus LiCl. Arrow marks animal death. Results are expressed as mean ± S.E. n = 3. *P < .05; **P < .01. B. DAI changes among the different groups after 5 days of DSS treatment. LiCl and AZD8055 (2.5 mg/Kg; [25]) were administrated the last 3 days. Results are expressed as mean ± S.E. n = 3. **P < .01. C. Representative image of colon photos of Ctl, DSS, DSS/LiCl and DSS/AZD8055 treated animals after 5 days of treatment. n = 3. Bar = 1 cm. D. Hematoxylin and eosin staining of colonic samples obtained from DSS and DSS/LiCl treated animals. Treatment was carried out for 5 days. Colitic mice received an intraperitoneal injection of LiCl the last 3 days. * = crypt abnormalities. Arrow = immune infiltration. Arrowhead = Ulcerated areas. n = 6. Bar = 500 μm. E. Effect of LiCl on histological scores in DSS-induced colitis for 5 days. LiCl administration was performed intraperitoneally every day. Results are expressed as mean ± S.E. n = 3. **P < .01.F. Effects of LiCl in the induction of necrosis in the colonic mucosa of DSS treated mice. Treatment was carried out for 5 days. Animals received intraperitoneal administration of LiCl for 3 days. Results are expressed as mean ± S.E. n = 3. ***P < .001.

mice and high-level of neutrophil and lymphocyte infiltration into the mucosal and submucosal areas were also noticed (arrows). The administration of LiCl worsened all those parameters. In fact, histological scores obtained by combining all these alterations corroborated that LiCl administration further enhanced mucosal damage during colitis (Figure 4E). In addition, the loss of cellular detail in contiguous cells and the augmented cellular debris that confirmed the presence of necroptosis after DSS treatment was augmented by LiCl (Figure 4F). Similar to the observed with LiCl, inhibition of mTOR by AZD8055 or mTORC1 by rapamycin enhanced epithelial damage and cell death in the mucosa of colitic mice as reported previously [22] (data not shown). Thus, our results strongly suggest that mTORC1 reduces IECs damage during colitis.

## mTORC1 Reduces DNA Damage in Epithelial Cells During Colitis

Next we investigated the mechanism by which mTORC1 prevented epithelial cell loss during colitis. mTORC1 reduces replicative stress and DNA damage response [9], thus we analyzed such possibility. To this purpose the presence of several DNA damage markers in the colonic mucosa of colitic mice exposed to LiCl or carrier alone was investigated. As shown in Figure 5A, colitis induction promotes the accumulation of the double-strand breaks (DSB) markers, pH2AX (γ-H2AX) and pp53, and the administration of LiCl further enhanced such process. Interestingly, neither DSS nor DSS/LiCl treatment affected the phosphorylation of CHK1, another DNA damage marker. Furthermore, IF staining for pH2AX revealed that LiCl treatment enhanced DNA damage in the colonic mucosa of DSS animals. Of note, DNA damage in the colonic mucosa of DSS and DSS/LiCl treated mice was detected at the same extent in both, epithelial and non-epithelial cells. (Figure 5B, arrow and arrowhead).

DSB in the mucosa of colitic mice as the ones observed here (pH2AX and pp53) are ROS-mediated [23] and our results suggest that mTORC1 prevented such process. Thus we investigated this

hypothesis by analyzing DSB in SW480 cells stimulated with IFNγ in the presence or absence of the mTORC1 inhibitors. As shown in Figure 5C IFNγ treatment reduced the phosphorylation of H2AX and p53 after 8 h of treatment. However, a strong increase in pH2AX and pp53 was noticed in monolayers stimulated with the cytokine plus the mTORC1 inhibitors. In addition, similar to the observed in our colitis model, pCHK1 levels were not affected by any of the treatments. IF staining demonstrated enrichment of pH2AX and pp53 at the nuclei of SW480 cells in all conditions (Figure 5D).

β-Catenin transactivation has been linked to DSB in epithelial cells [24] and IFNγ inhibits β-catenin co-transcriptional activity in IECs [2,3]. Thus we speculated that mTORC1 could be affecting β-catenin transactivation and therefore, we analyzed the presence of EGFP in the mucosa of LGR5-EGFP colitic mice treated with rapamycin. As shown by the presence of EGFP, β-catenin transactivation was enhanced after mTORC1 inhibition (Figure 5E) and this process occurs in at least two cell populations present in the mucosa of colitic mice (Figure 5F, arrows and red asterisk). EGFP presence correlated with the increase in the proliferation marker pHist3 in epithelial cells (Figure 5, F and G, (arrows)). However, the expression of EGFP was more prominent in non-epithelial cells that were always negative for pHist3 (Figure 5F, red asterisk). Although those cells were always located in close contact with epithelial cells present at the crypts. Furthermore, TOP-Flash assays in SW480 demonstrated that rapamycin and LiCl treatment reversed the inhibition in β-catenin co-transcriptional activity that was induced by IFNγ. Rapamycin alone did not affect β-catenin co-transcriptional activity but as expected from previous reports [24] LiCl alone strongly enhances such process (Figure 5H). Because of these results we analyzed the presence of the so called Active β-catenin (ABC) in the mucosa of colitic mice after mTORC1 inhibition. Interestingly, no changes in cellular distribution or protein levels for the molecule were noticed after DSS or DSS plus LiCl treatment when compared with control animals (Figure 5, I and J). Similar results were observed after inhibiting mTORC1 with rapamycin in DSS-treated mice (data not shown). However, ABC was present at the cytosol of colonic cells in all

Figure 5. mTORC1 prevents DNA damage in IECs during colitis. A. Effects of LiCl in the induction of DNA damage in the mucosa of colitic mice. pγ-H2AX, pp53 and pCHK1 were evaluated by Western blot. DSS treatment was carried out for 5 days. LiCl was administered intraperitoneally every day the last 3 days. GAPDH was used as loading control. n = 3. B. Immunofluorescence staining for pγ-H2AX was performed in frozen sections of colitic mice. DSS treatment was carried out for 5 days and LiCl was administered intraperitoneally for 3 days. E-cadherin = red. pγ-H2AX = Green. Nuclei = blue. Bar = 50 μm. n = 3. C. Effects of LiCl, AZD8055 and Rapamycin in the induction of DNA damage in SW480 cells treated with IFNγ. pγ-H2AX, pp53 and pCHK1 were evaluated. IFNγ treatment was carried out for 8 h. LiCl, AZD8055 and Rapamycin were added 30 min before IFNγ treatment. GAPDH was used as loading control. n = 3. D. Immunofluorescence staining for pγ-H2AX (green) and pp53 (red) was performed in SW480 cells treated with IFNγ. LiCl, AZD8055 and Rapamycin were added 30 min before IFNγ treatment. IFNγ treatment was carried out for 8 h. Nuclei = blue. Bar = 20 μm. n = 3. E. Effects of Rapamycin in the induction EGFP expression in the mucosa of LRG5-EGFP colitic mice. EGFP and pS6 were evaluated by Western blot. DSS treatment was carried out for 5 days. Rapamycin was administered intraperitoneally every day during the last 3 days. Actin was used as loading control. n = 3. F, G. Immunofluorescence staining for pHist3 (Red) and EGFP (Green) was performed in frozen sections of LRG5-EGFP that were induced to colitis. DSS treatment was carried out for 5 days and LiCl was administered intraperitoneally for 3 days. Dash line marks crypt base. White arrow = IEC double positive. Red asterisk = non-epithelial cells GFP positive. Nuclei = blue. Bar = 20 μm. n = 3. H. β-catenin transactivation was evaluated *in vitro* by TOP-Flash assay. SW480 cells were treated with IFNγ for 18 h. LiCl, AZD8055 and Rapamycin were added the last 6 h of IFNγ treatment. Fop-Flash was used as negative control. Values were normalized to CMV-renilla. Graphs represent single experiment performed by triplicate. Data are expressed as mean ± SEM. n = 2. **P < .01. I. ABC and total β-catenin were analyzed in the mucosa of colitic mice, LiCl administration was performed intraperitoneally for 3 days. DSS was administered in tap water for 5 days. n = 3.J. Immunofluorescence staining for ABC (Green) and F4/80 (Red) was performed in the mucosa of Ctl, DSS and DSS/LiCl treated mice. In mice, LiCl administration was performed intraperitoneally for the last 3 days. DSS was administered in tap water for 5 days. Nuclei = blue. n = 3. Bar = 20 μm. K. pγ-H2AX and total β-catenin were analyzed in cell lysates of RKO cells treated with Doxorubicin (3.5 μM). IFNγ, LiCl and AZD8055 were added 30 min before DOX treatment. Dox treatment was carried out for 12 h. Actin was used as loading control. n = 3. L. iNOS, total β-catenin and IL-1β were analyzed in cell lysates of J774 cells treated with mIFNγ (250 μg/ml)/LPS (25 ng/μl) for 12 h. LiCl, AZD8055, Rapamycin and XAV939 were added 30 min before IFN/LPS treatment. Stimulation was carried out for 12 h. GAPDH was used as loading control. n = 3.
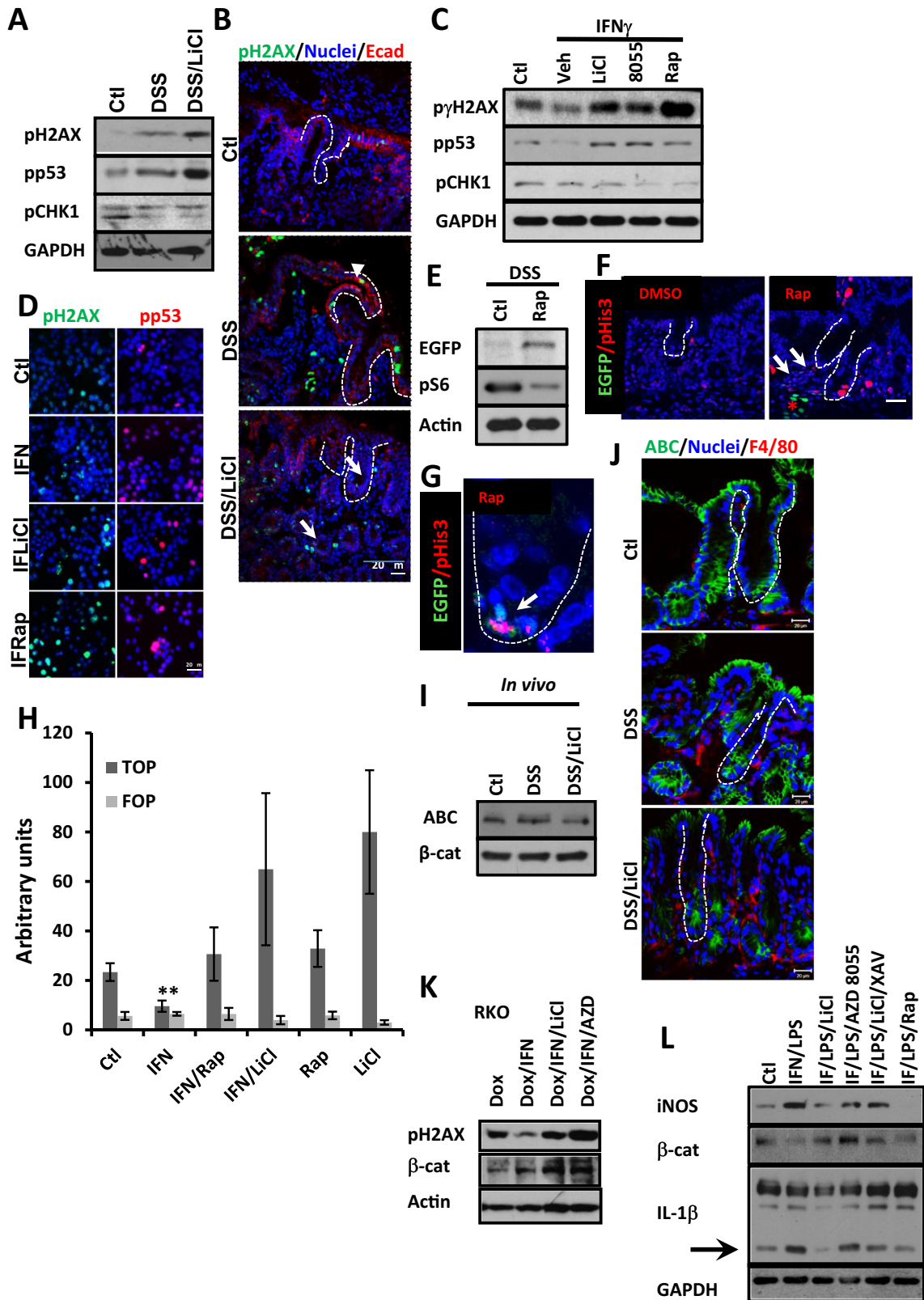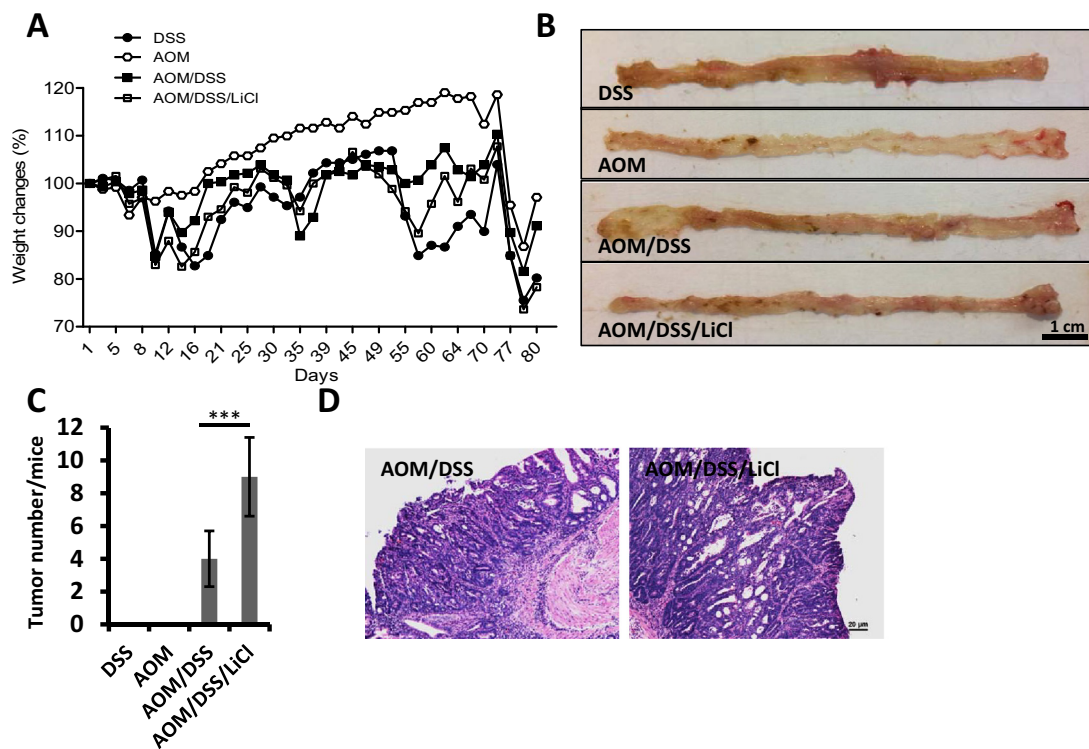
Figure 6. mTORC1 inhibition exacerbates epithelial transformation during colitis. A. Percentage of body weight change in DSS, AOM, AOM/DSS and AOM/DSS/LiCl treated mice. Graphs represent single experiment performed by duplicate. Data are expressed as mean ± SEM. n = 4. B. Representative image of colon photos of DSS, AOM, AOM/DSS and AOM/DSS/LiCl treated mice. n = 4. Bar = 1 cm. C. Quantification of tumors present at the colonic mucosa of mice treated with DSS, AOM, AOM/DSS and AOM/DSS/LiCl. Results are expressed as mean ± S.E. n = 4. ***$P < .001$. D. Hematoxylin and eosin staining of colonic tumors obtained from AOM/DSS and AOM/DSS/LiCl treated mice. n = 6. Bar = 20 μm.

conditions and was clearly detected in the nuclear samples of colitic mice that were exposed to LiCl (Supplementary Figure 3A). Total β-catenin also was enriched at the cytosolic compartment in all conditions but, the molecule was detected at the nuclei of control animals and its presence was increased after colitis induction as previously reported [25]. LiCl administration to DSS-treated mice further enhanced β-catenin accumulation at nuclear fraction, strongly supporting that mTORC1 inhibits β-catenin co-transcriptional activity. In agreement with our previous published results [3] mTORC1 inhibition by LiCl or Rapamycin increased ABC protein levels in SW480 cells that were stimulated with IFNγ. Of note, ABC staining was observed in the cytosol of macrophages (F4/80+) present at the colonic mucosa of DSS and DSS/LiCl treated mice (Supplementary Figure 3B, white arrow).

Our results prompted us to speculate that inhibition of β-catenin co-transcriptional activity plays a protective role in the generation of DSB in IEC. To address this hypothesis we evaluated the presence of DSB in SW480 cells exposed to Doxorubicin (Dox), a drug that triggers DNA damage [26], in presence of IFNγ or the β-catenin inhibitor, XAV939. As expected from previous results, the administration of IFNγ strongly reduced the DNA damage induced by Doxorrubicin as shown by the presence pH2ax (Figure 5K). Inhibition of mTORC1 activity by LiCl or AZD8055 blocked the protective effect in the formation of DSB that was elicited by IFNγ. Furthermore, in agreement with our previous results mTORC1 inhibition by LiCl or AZD8055 resulted in increased presence of β-catenin in our epithelial cells. Similar results were observed in SW480 cells (Supplementary Figure 4B). Next we investigated the role of mTORC1 in the function of macrophages. To this end macrophage cell

line J774 was stimulated with the mix IFNγ/LPS, a well-known process that induces mTORC1 activation in those cells [27,28], in the presence or absence of mTORC1 inhibitors. As expected, IFN/LPS stimulation induced the expression of iNOS and the processing of IL-1β (arrow) and also reduced β-catenin protein levels. However, administration of LiCl, AZD8055 or Rapamycin strongly reversed both effects. The administration of the β-catenin inhibitor, XAV939, prevented the reduction in iNOS and IL-1β stimulated by LiCl and also reduced of β-catenin protein levels (Figure 5L). mTORC1 inhibition by Rapamycin yielded similar results in the commitment of M2 Bone Marrow Derived Macrophages (BMDM) to the M1 phenotype by LPS/IFNγ (Supplementary Figure 4C). Thus, taken together, our data strongly suggests that mTORC1 inhibits β-catenin co-transcriptional activity during colitis in order to prevent epithelial DNA damage and this process also favors the induction of a proinflammatory phenotype in macrophages.

mTORC1 Inhibition Enhances Tumor Formation in an AOM/DSS Murine Model of Colon Carcinogenesis

DNA damage during colitis and anti-inflammatory responses has been associated with development of colon carcinogenesis [23,29,30]. Thus we next analyzed the effect of LiCl administration in the tumor formation using a murine model of colon carcinogenesis associated to colitis. The protocol for treatment is depicted in Supplementary Figure 4A. Analysis of weight loss demonstrated that AOM/LiCl, DSS/LiCl, DSS/AOM and DSS/AOM/LiCl treated mice displayed very similar weight changes during the whole course of the experiment (Figure 6A). Furthermore, as shown in Figure 6B tumor formation was only detected

in DSS/AOM and DSS/AOM/LiCl treated animals but not in AOM or DSS administered mice. As expected from our previous results demonstrating that LiCl enhances proliferation and DNA damage in IECs the number and size of the tumors greatly increased in the mucosa of DSS/AOM/LiCl when compared with DSS/AOM (Figure 6C). Histologically in both conditions, we observed a moderately differentiated adenocarcinoma characterized by the presence of epithelial cells with ovoid and enlarged nuclei, also numerous mitotic bodies were noticed (Figure 6D). Thus taken together our results strongly suggest that inhibition of mTORC1 during colitis strongly contributes to colorectal cancer development and growth.

Discussion

During colitis IFNγ plays an important role in the disruption of intestinal homeostasis by suppressing β-catenin-mediated cell proliferation [3,25,31]. The mechanisms associated with these alterations are not well characterized. Here, we provide strong evidence that the molecular machinery implicated in the inhibition of cell proliferation involves mTORC1 activation. Using an inflammatory model, we demonstrated that IFNγ triggered mTORC1 signaling in IECs along the crypt axis during inflammation. mTORC1 activation during colitis prevented the proliferation of IECs and its inhibition with LiCl or rapamycin reversed such process. In that context both treatments promoted pHist3 phosphorylation and LiCl administration clearly stimulated EdU incorporation and the generation of new IECs. Interestingly, pharmacological inhibition of mTORC1 also resulted in a high rate of DNA damage. Accordingly, mTORC1 inactivation by MCRS1 or Rheb ablation also resulted in high epithelial cell death due to high levels of DNA damage [9,22]. Thus the results provided here support at least two mechanisms that directly contribute to those findings. 1. mTORC1 activation directly prevents DNA damage in IEC by inducing the activation of DSB reparation machinery [32] and 2. mTORC1 inhibits the proliferation of stem cells and therefore reduced DNA damage. We believe mTORC1 activation could be necessary for proper completion of cell cycle and it could play a role in the DNA damage checkpoints. Furthermore, given that autophagy activation accelerates stem cell division and hyperproliferation [33], inhibition of autophagy by mTORC1 [34] could be involved in the safeguard of the intestinal stem cell niche. Thus mTORC1 activation during colitis could be part of a mechanism to slow down the proliferative rate of IECs that are living in a harsh environment such as the one created by the high levels of ROS [35]. Such process could be especially important to reduce the rate of DSB in the nuclear DNA of in intestinal epithelial stem cells during inflammation. Elucidating the complete role of mTORC1 in such system might be particularly important for understanding the mechanics and pathophysiology of CRC associated to colitis.

During colitis LiCl treatment was more efficient to induce cell proliferation in IECs than rapamycin, thus autophagy mTORC1 dependent and mTORC1 independent [36] may play similar role in activation of IEC proliferation in the intestinal epithelium. However, given that LiCl modulates several other signaling pathways implicated in the activation of cell proliferation in epithelial cells (*e.g.* inactivation of GSK3β) [37–39] we cannot rule the existence of another mechanism. In fact the dependence of ABC in the induction of IECs proliferation and DNA damage observed here strongly support the last scenario. Therefore, LiCl could be defined as a broad inhibitor that targets several signaling pathways activated downstream of IFNγ

and represents a unique tool that could help to elucidate the role of IFNγ in the maintenance of IEC homeostasis during inflammation.

Inhibition of cell proliferation during colitis leads to ulcer formation and epithelial barrier breakdown [4]. However, our results strongly suggest that could also represent a unique adaptive mechanism aimed to protect IEC from further damage during inflammation. The nature and function of such mechanism needs to be investigated; but it is tempting to speculate that it might be targeting the stem cell compartment and therefore could be important in the regulation of epithelial restitution and cancer development, two events that have been observed during chronic colitis [23,30]. The mechanism underlying such process needs to be further investigated but our results suggest that transactivation of β-catenin in epithelial cells and in macrophages could play an important role. Accordingly, β-catenin enhances intestinal epithelial cell proliferation [40] and in the absence of E-cadherin, a well-known β-catenin inhibitor, necrosis and anoikis sparked in IECs during colitis [41,42]. Such process could be directly related to the hyperproliferation induced by β-catenin. However, we cannot rule out the possibility that transactivation of β-catenin after mTORC1 inhibition could stimulate the production of soluble factors in IECs, that in turns will stimulate the recruitment of M2 macrophages (IL-6$^+$) at the inflamed epithelium as described previously [29]. Furthermore, giving that mTORC1 is necessary to induce the commitment of macrophages into a proinflammatory phenotype [28], it could be easy to understand why rapamycin and LiCl administration could also result in the generation of an anti-inflammatory environment mediated by M2-like macrophages. M2-macrophages in the colonic mucosa will in turn generate an ideal micro-environment that will stimulate CRC development as previously reported [29]. In agreement with this hypothesis recent evidence demonstrates that Wnt/β-catenin activation in macrophages as the one achieved after mTORC1 inhibition, promotes alternative M2-activation in macrophages [43,44] and mTORC1 positively regulates IL6/STAT3 signaling a complementary signaling pathway associated with Wnt/β-catenin in cancer development [9,22]. Thus uncontrolled activation of convergent signaling pathways could be stimulated by mTORC1 inhibition and this process will enhances not only epithelial damage but proinflammatory environments that contribute to cancer development.

The mechanism by which mTORC1 inhibits cell proliferation of IECs needs to be further investigated. However, given that Asano et al., demonstrated that inhibition of autophagy severely impairs intensive regeneration in IECs [33], the activation of mTORC1 by IFNγ could be involved in modulating such process. In agreement with this hypothesis Raup-Konsavage et al. demonstrated that LiCl, a drug that triggers autophagy [36,45] promotes colonic regeneration during the recovery period from acute DSS-induced colitis [39]. Thus, mTORC1 activation downstream of PI3K/Akt signaling could be important to inhibit autophagy and cell proliferation during inflammation. However inhibition of β-catenin transactivation and its effects in the regulation of soluble mediators known to positively regulated proliferation and anti-inflammatory responses, such as IL-10 needs to be evaluated [29]. mTORC1 then could be envisioned as part of the machinery that prevents uncontrolled proliferation in hostile environments and therefore could be essential to prevent DNA damage in stem cells that otherwise would stimulate cancer development.

In conclusion, we showed that mTORC1 is activated by IFNγ in the colonic mucosa during inflammation and inhibits cell

proliferation, prevents epithelial damage, stimulates a proinflammatory environment and reduces mortality and CRC development in a model of colitis DSS-induced.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.08.016.

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