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Rapamycin extends life span in *Apc*$^{Min/+}$ colon cancer FAP model

Manish Parihar[1], **Sherry G. Dodds**[1], **Gene Hubbard**[2], **Marty Javors**[2,5], **Randy Strong**[4,6], **Paul Hasty**[1,2,3,*], **Zelton Dave Sharp**[1,2,3,*]

[1]Department of Molecular Medicine and Institute of Biotechnology, University of Texas Health, San Antonio, Texas 78245.

[2]Barshop Institute for Longevity and Aging Studies, University of Texas Health, San Antonio, Texas 78245.

[3]Mays Cancer Center, University of Texas Health, San Antonio, Texas 78245.

[4]Department of Pharmacology, University of Texas Health, San Antonio, Texas 78245.

[5]Department of Psychiatry, University of Texas Health, San Antonio, Texas 78245.

[6]San Antonio Geriatric Research, Education and Clinical Center, University of Texas Health, San Antonio, Texas 78245.

Abstract

Micro: Rapamycin extends life and health span in wild type mice suggesting prevention of cancer. We show rapamycin extends survival in colon cancer prone mice and that crypt cells are primary targets of mTOR inhibition that mediate tumor prevention or delay.

Structured: We previously showed that lifelong rapamycin treatment of short lived *Apc*$^{Min/+}$ mice, a model for familial adenomatous polyposis (FAP), resulted in a normal lifespan. *Apc*$^{Min/+}$ mice develop colon polyps with a low frequency but can be converted to a colon cancer model by dextran sodium sulfate (DSS) treatments (*Apc*$^{Min/+}$-DSS model). We asked, what effect would pretreatment of *Apc*$^{Min/+}$ mice with chronic rapamycin prior to DSS exposure have on survival and colonic neoplasia? Forty-two ppm eRapa diet exacerbated the temporary weight loss associated with DSS treatment in both sexes. However, our survival studies showed that chronic rapamycin treatment significantly extended lifespan of *Apc*$^{Min/+}$-DSS mice (both sexes) by reductions in colon neoplasia and prevention of anemia. Rapamycin also had prophylactic effects on colon neoplasia induced by azoxymethane (AOM) and DSS in C57BL/6 males and females. Immunoblot assays showed the expected inhibition of mTORC1 and effectors (S6K→rpS6 and S6K→eEF2K→eEF2) in colon by lifelong rapamycin treatments. To address the question of cell

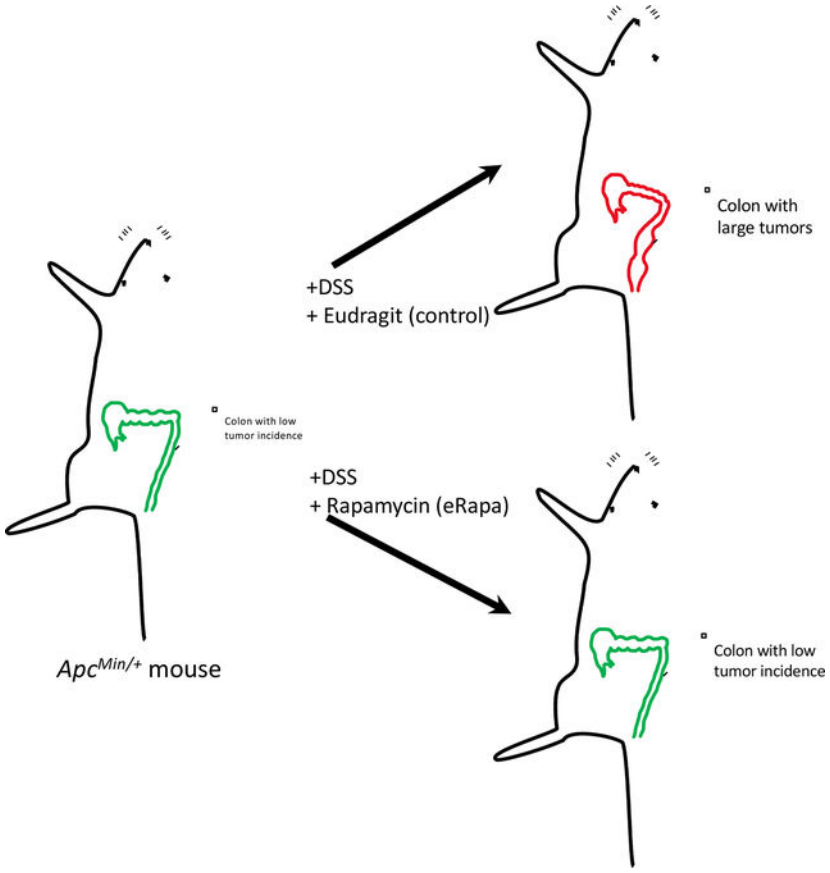
*Corresponding authors: Zelton Dave Sharp, sharp@uthscsa.edu, 210.667.7465, Fax, 210.567.7277 and Paul Hasty, hastye@uthscsa.edu.

Conflict of Interest:

Under a licensing agreement between Emtora Biosciences, Inc. and the University of Texas Health Science Center San Antonio, Z.D. Sharp, P. Hasty, R. Strong and the University are entitled to milestone payments and royalty on sales of the rapamycin formulation used in this paper.

types affected by chronic enteric rapamycin treatment, immunohistochemistry analyses demonstrated that crypt cells had a prominent reduction in rpS6 phosphorylation and increase in eEF2 phosphorylation relative controls. These data indicate that enteric rapamycin prevents or delays colon neoplasia in $Apc^{Min/+}$+DSS mice through inhibition of mTORC1 in the crypt cells.

Graphical Abstract



Micro Abstract:

Rapamycin extends life and health span in wild type mice suggesting prevention of cancer. We show rapamycin extends survival in colon cancer prone mice and that crypt cells are primary targets of mTOR inhibition that mediate tumor prevention or delay. *Structured*: We previously showed that lifelong rapamycin treatment of short lived $Apc^{Min/+}$.

Keywords

aging; rapamycin; mTORC1; crypt stem cells

Introduction:

Survival and growth of cancer cells depend upon the maintenance of an anabolic state. The mechanistic (also mammalian) target of rapamycin (mTOR) kinase is central for this maintenance. Oncogenic signaling such as PI3K-Akt captures mTOR complexes 1 and 2

(mTORC1 and mTORC2) to promote cancer's anabolic state. Accordingly, hyperactive mTOR signaling is present in about 70% of human cancers[1] and is sufficient to cause hepatocellular carcinoma in mice[2]. For cancer cell growth (in mass), mTORC1 promotes biosynthesis of proteins, lipids and nucleic acids[3]. Because of its central role in cancer cell growth and proliferation, much attention has been directed toward development of drugs that inhibit mTOR, primarily mTORC1. Rapamycin (and closely related rapalogs), the first-generation drugs that allosterically inhibit mTORC1 (and long term mTORC2 *in vivo*) was followed by second and third generation mTOR kinase inhibitors. These latter generation active site mTOR inhibitors (asTORi) reduce the activities of both mTOR complexes and are being evaluated as cancer treatments in recent clinical trials. Resistance due to rebound Akt activity in the absence of mTORC2 input[4–6] represent a hurdle for the asTORi class of drugs.

The Intervention Testing Program (ITP) showed that chronic rapamycin treatments started late or mid-life in UM-HET3 genetically heterogeneous male and female mice resulted in a maximum extension of life span[7–9]. A small molecule that extends maximum life span in UM-HET3 mice would accomplish this either by preventing, delaying and/or reducing the severity of cancer. Thus, although rapamycin has proven to be only modestly effective as a cancer treatment[10], preclinical studies in wild type mice indicate it might be effective as a preventive. This conjecture has been tested in several cancer-prone models, including mice susceptible to intestinal tumorigenesis as observed in those harboring mutations in the adenomatous polyposis coli (*Apc*) gene.

$Apc^{Min/+}$ mice[11] model the polyposis observed in FAP, including multiple adenomas in the intestine, intestinal bleeding and anemia[12]. $Apc^{Min/+}$ mice have been used extensively in preclinical cancer prevention and therapeutic settings. Relevant to this study, Fujishita et al. showed that polyps in $Apc^{\Delta 716}$ mice have elevated mechanistic target of rapamycin (mTORC1) pro-growth activity, which was reduced by treatment with the mTOR inhibitor RAD001[13]. We showed that chronic treatment of $Apc^{Min/+}$ mice with an enteric formulation of rapamycin (eRapa) reduced polyposis in the small intestine leading to a five-fold extension of life span and a mean life span that was greater than wild type[14]. A major contributor to this extension of life and health span in this model was normalization of hematocrits.

As a model for FAP, $Apc^{Min/+}$ mice are not optimal since they develop large bowel neoplasms at a low frequency making them less attractive as a model for human colon cancer. One method to convert $Apc^{Min/+}$ mice into a human-relevant colon cancer model is to treat them with dextran sodium sulfate (DSS), which greatly increases incidence, multiplicity and size of tumors[15,16]. By week 3 after DSS challenge, 100% of $Apc^{Min/+}$ mice have colonic adenomas, and by week 5, 100% have colonic adenocarcinomas[16]. We asked, would chronic enteric delivery of rapamycin prevent large bowel neoplasms and how would this affect survival in the $Apc^{Min/+}$-DSS model?

Materials and Methods

Mice, eRapa chow, and rapamycin blood levels.

We treated and housed mice according to Institutional Animal Care and Use Committee standards and the NIH guide for care and use of lab animals. We obtained cohorts of $Apc^{Min/+}$ male and female mice from Jackson Laboratories and fed them diet containing 42 ppm microencapsulated rapamycin[17] which provided a dose of 6.72 mg of rapamycin /kg body weight /day. Control diet was same but with empty Eudragit capsules instead of eRapa. We started both diets at four weeks of age. We previously described preparation of rapamycin diets in Livi et al.[18]. We started DSS treatment (2.5% in drinking water) when the animals were 8 wks old and administered it for one week, after which it was replaced with drinking water. For cross-sectional studies, we sacrificed the animals at 16 weeks of age to harvest tissue and blood.

C57BL/6 male and female mice obtained from Jackson Laboratories underwent the AOM/DSS colon cancer induction procedure[15]. We started eRapa or Eudragit at 8 weeks of age and gave a single dose (12 mg/kg bw) of AOM by IP injection at 12 weeks of age. DSS (2.5% in drinking water) was given in 3 cycles of: 7 days on DSS followed by 14 days on water for recovery. The first cycle was started 3 days after AOM, and animals were sacrificed for hematocrits and tissues at the end of the third cycle. We determined rapamycin blood level as described previously[18] and for survival studies, mice were allowed to live out their lifespan; i.e. we did not censor due to morbidity. Mice were only euthanized if they either 1) were not able to eat or drink, 2) were bleeding from a tumor or other condition, or 3) were laterally recumbent failing to right themselves or to move when prodded.

Pathology

We assessed hyperplasia and neoplastic lesions using the grading system previously described[19]. A pathologist separately examined the specimens without knowledge of the diet or sex. We used the following grading scale:

Diagnois Grade	Grade Score
Hyperplasia 1	1
Hyperplasia 2	2
Hyperplasia 3	3
Hyperplasia 4	4
Neoplasia 1	5
Neoplasia 2	6
Neoplasia 3	7
Neoplasia 4	8

Immunoblots

We performed immunoblots as described previously[14]. Additional antibodies used were mouse anti gapdh (1:2000, Santa Cruz Biotechnology, sc-365062) and rabbit anti eEF2k (1:800, Cell Signaling Technology; 3692).

Hematocrits

We collected blood at the time of sacrifice by cheek bleeding. Cells from 50–75 μL of whole blood were packed by centrifugation in heparinized micro-hematocrit capillary tubes (Fisherbrand cat. 22-362-566).

Immunohistochemistry

Tissues were fixed in 10% formalin for 24–48 hours at room temperature, transferred to 70% ethanol, embedded in paraffin, and sectioned. Antigen retrieval was performed by heating at 95–100°C in sodium citrate buffer (10mM sodium citrate, 0.05% tween 20 at pH 6.0) using a microwave. Endogenous peroxidase activity was blocked by dipping in 3% hydrogen peroxide for 10 mins. Sections were then blocked using 5% normal goat serum followed by overnight incubation at 4°C with primary antibody in a humid chamber. Signal was detected using Cell Signaling Technology's (CST) Signal Stain Boost IHC Detection Reagent and Signal Stain DAB Substrate Kit (CST 8059). Slides were then counter stained with hematoxylin (CST 14166). Images were taken using the Echo Revolve FL microscope. Antibodies were rabbit anti-rps6 (1:600; 2217) and rabbit anti-phospho-rps6 ser240/244 (1:2000; 5364) from CST and rabbit anti-Phosho (Thr56)-eEF2 (1:500; Invitrogen PA5–38085).

Results

To address the question of rapamycin effects on DSS induced carcinogenesis in $Apc^{Min/+}$ female and male mice, we fed mice a diet containing 42 ppm enteric (encapsulated) rapamycin (eRapa). This was the optimal life extending dose in our previous study of $Apc^{Min/+}$ females[14]. Control diet contained empty Eudragit capsules.

To assess life span effects by chronic eRapa, we conducted survival studies of the DSS-treated $Apc^{Min/+}$ males and females. Fig 1 results showed a significant increase in lifespan afforded by the rapamycin-containing diets (RD) compared to Eudragit diets (ED) in both sexes.

We next observed effects on carcinogenesis in DSS-treated mice by cross section studies at 16 weeks, Fig 2a, groups A and B. Figs 2b and c show images of typical large bowels from DSS-treated $Apc^{Min/+}$ mice fed control or eRapa diets, respectively. The colon in Fig 2b is typical of the heavy tumor burden resulting from the colitis inflicted by DSS treatment. Conversely and strikingly, Fig 2c shows an example of the almost complete prevention of this carcinogenesis by chronic treatment with eRapa. We observed no polyposis or tumors in eRapa-treated (no DSS) $Apc^{Min/+}$ mice (Fig 2d). Table 1 summarizes colon tumor counts with ED groups being too numerous to count (TNC) while RD groups were greatly reduced.

As predicted, large intestine from non-DSS $Apc^{Min/+}$ mice showed minimal tumor incidence, which eRapa further reduced to zero in this study (Table 1).

We next graded tumor progression using Grade Scores (see methods for grading scale) on random sections prepared from $Apc^{Min/+}$ mice. Pathology analysis of female $Apc^{Min/+}$ mice showed a range of 1, 8, 7, 0 and 0 for ED mice, and 7, 2, 3, 6 and 1 for RD mice. There were no colon tumors in non-DSS treated female $Apc^{Min/+}$ mice for either ED or RD mice. Male ED $Apc^{Min/+}$mice showed Grade Scores of 5, 6, 7, 8 and 7, while RD males had Scores of 6, 8, 1, 0 and 0. One Eudragit non-DSS male had a score of 8, while the remainder, including eRapa treated, scored 0. Thus, while rapamycin pre-treatment greatly reduced tumor incidence, it did not appear to affect the grade of tumors that developed in these mice.

Graphs in Fig 3a and b compare weights of ED and RD female and male $Apc^{Min/+}$ mice, respectively. Each group demonstrated the characteristic loss in weight subsequent to one week of DSS-containing water. RD females and males showed a similar initial reduction in weight (22%, females and 23% for males). Interestingly, this initial loss is twice that of ED animals. We do not know the reason for this difference. Whatever the cause, it did not appear to affect either sex's ability to regain weight after going back on water without DSS. These graphs also illustrate eRapa treatment preventing the dramatic weight loss after week 12 in ED animals due to illness resulting in short lifespan (Fig 1). Furthermore, Fig 3c shows the typical reduction of hematocrits in $Apc^{Min/+}$ mice that is reversed or prevented by chronic rapamycin-containing diets, which likely also contributed to the extended longevity of the RD group (Fig 1).

$Apc^{Min/+}$ mice carry a mutated copy of *Apc* gene and suffer from both polyposis and anemia. Accordingly, we wanted to find out if rapamycin would be as effective in wildtype mice made susceptible to colon cancer by another approach such as chemical carcinogens and tumor promotion. For this purpose, we chose the azoxymethane (AOM) DSS approach described by Rosenberg[15]. For wild type mice, we used C57BL/6 (same strain as $Apc^{Min/+}$) treated with AOM and DSS as described in Materials and Methods. Rapamycin-containing diet (42 ppm) in a prevention setting was effective in reducing colon tumors significantly in both females and males (Fig 4 a and b). A significant difference in these animals is that neither experimental group developed anemia (Fig 4c and d) despite clearly visible blood in their stools. Chronic rapamycin had no effect on hematocrits or bloody stools, suggesting its positive effects on $Apc^{Min/+}$ anemia is due to *Apc* heterozygosity effects in other organs.

Since the anabolic state of most cancers depends on active mTORC1[1,2], it is important to assess the effects of chronic inhibition by rapamycin in the $Apc^{Min/+}$-DSS model. For this purpose, we harvested large bowel after 12 weeks of eRapa or control diets (see Fig 2a for experimental design), and prepared tissue lysates for immunoblot assays. Fig 5 shows typical blot images and graphs of signal intensities for both female and male mice. Below each blot we show rapamycin concentrations in blood with females having higher blood concentrations than males as observed in other studies documenting sexual dimorphism in blood levels of RD animals[9] Miller et al.[9] discuss potential reasons for this difference and suggest it is most likely due to differential response of females to rapamycin rather than differences in consumption of food containing equal concentrations of rapamycin.

Regardless of these pharmacokinetic differences, both females and males showed significant decreases in mTORC1 activity as assessed by reductions in Ser 240/244 phosphorylated ribosomal protein S6 (rpS6) relative to rpS6 protein (Fig 5 a, b, and d, e, respectively). We observed no significant differences for Akt rebound in response to eRapa treatment as seen by immunoassayed Ser473-phospohorylated (by mTORC2)-Akt (Fig 5 a, c and d, f, females and males respectively).

We next wanted to understand where chronic rapamycin has its prevention effects in colon. Fig 6a shows IHC image of a large tumor in a DSS/Eudragit treated $Apc^{Min/+}$ female colon compared to an eRapa-treated colon devoid of detectable tumors in this image (Fig 6c). Note that the small tumors we detected by touch in eRapa treated DSS colon are rare and difficult to find in sections. We observed prominent levels of phospho-rpS6 in the tumors, which is consistent with increased mTORC1 activity (Fig 6 a, b). Cells in the crypts of Eudragit-DSS treated colon also showed higher levels of phospho-rpS6, which was undetectable in the eRapa-DSS treated animals (Fig 6d, e). Phosphorylation-independent (total) rpS6 however, showed little if any change in the signal intensity with eRapa treatment (Fig 6 f, g). Comparing Figs 6d with 6c illustrates the high level of mTORC1 activity in tumors relative to levels in surrounding normal tissues. Together, these data indicate that chronic rapamycin exposure in $Apc^{Min/+}$ colon results in mTORC1 inhibition in crypt cells.

Because rapamycin preferentially inhibits S6K over 4EBP1 in a liver regeneration setting[20], the mTORC1–4E-BP pathway might not be the limiting axis in tumor promotion and growth. While translation initiation control by mTORC1 has been extensively studied, mTOR effects on translation elongation has drawn less attention. In anabolic settings, S6 Kinase 1 (S6K)-dependent phosphorylation eukaryotic elongation factor 2 (eEF2K) inhibits its activity[21]. Upon mTORC1 (and S6K) inhibition, dephosphorylation of these sites frees eEF2K to phosphorylate eukaryotic elongation factor 2 (eEF2), which results in reduced translation elongation. Interestingly, Faller et al. showed that, in addition to mTORC1 effects on protein synthesis and tumor growth in $Apc^{f/f}$ mice by way of S6 kinase (S6K), translation elongation is also a significant factor[22]. To assess the effects of chronic eRapa on this in the $Apc^{Min/+}$-DSS model, we first used immunoblot assays to determine eEF2K levels. In both females and males, rapamycin increased the levels eEF2K levels significantly in large intestine lysates (Fig 7a–d). Using antibodies specific for Thr56-phosphylated eEF2, immunohistochemistry assays showed an increase in phosphorylation (Fig 7f and h) compared to Eudragit controls (Fig 7e and g) indicating a reduction in elongation in eRapa treated males and females.

Discussion:

Based on our preclinical findings, chronic enteric delivery of rapamycin looks promising as a prophylactic in colon cancer. Previously we showed that chronic eRapa-diets provided a normal lifespan for $Apc^{Min/+}$ female mice that, without treatment, die of anemia at about 180 days (a fivefold increase in survival)[14]. In this study, the reduction in tumor incidence in the $Apc^{Min/+}$–DSS model by chronic rapamycin diets is consistent with other cancer prevention studies[13,14,18,23–25]. In agreement with Faller et al., data on small intestine in $Apc^{f/f}$ mice, we report evidence supporting the idea that chronic inhibition of mTORC1

inhibits translation elongation also in large intestine crypts of *Apc*$^{Min/+}$–DSS mice. These two hits on protein synthesis likely contribute significantly to the dramatic reduction in tumors we observe in a setting of chronic rapamycin treatment.

The increase in longevity by chronic rapamycin in the *Apc*$^{Min/+}$-DSS model is also consistent with the survival results in previous *Apc*-deficient model studies[13,14]. To achieve these increased lifespan results, we think that rapamycin is working on multiple levels to improve survival in *Apc*$^{Min/+}$-DSS mice. First, based on cross section studies, tumor burden is greatly reduced. Second, anemia is prevented. Third, chronic rapamycin also delays, prevents or reduces the severity of other diseases associated with aging in these mice. This represents “compression of morbidity”[26], one of the major goals of cancer and aging research. It is curious that chronic rapamycin treatment seems to work better at preventing cancer than treating it. In this respect it mimics another well-known anti-aging intervention – diet restriction[27].

The eRapa formulation is designed to release the drug at a neutral pH, which in mice is in the vicinity of the distal small intestine[14] and large bowel[28] based on levels of mTORC1 inhibition. However, the cell type in colon that rapamycin targets was not known. Our IHC data lead us to posit that a main cell type which chronic rapamycin affects is the Paneth-like goblet cells in the colonic crypts. This conjecture is based on Rothenberg’s proposal that Paneth-like *cKit*$^{+}$ goblet cells are thought to perform the same function in colon as Paneth cells do in small intestine; i.e. support *Lgr5*$^{+}$ intestinal crypt stem cells[29]. Yilmaz, et al.[30] proposed that Paneth cells in the small intestine promote stem cell renewal in response to calorie restriction and rapamycin. Thus, Paneth-like cells in the large intestine might also perform the same function, that is support *Lgr5*$^{+}$ stem cells. As observed in small intestine crypt stem cells by Yilmaz et al.[30], chronic rapamycin may promote Lgr5^{+} stem cell self-renewal in colon, which we posit helps prevent large intestine cancer in the *Apc*$^{Min/+}$-DSS model.

While DSS in *Apc*$^{MIn/+}$ mice is useful for a colon cancer-prone model, its use also introduces caveats in interpreting our results. Previous studies have shown that there is a homeostasis in the crypt through interactions between the Paneth-like and the stem cells[31]. In an inflammation-based model like DSS there is a loss of several types of cells, including colon Lgr5^{+} stem cells[32]. Additionally, there is de-differentiation of other cells types (e.g., Paneth) to replenish lost stem cells[32]. Thus, the dynamic changes in the crypts of *Apc*$^{Min/+}$ mice treated with DSS may explain our unsuccessful attempts to use known cell markers to precisely determine if Paneth-like cells are targeted by eRapa in this model. Cell lineage tracing studies[29] will likely be required to properly identify the target cell. What we do know is that the rapamycin effect appears to be concentrated in the crypt, with Paneth-like cells being the most likely candidate.

In *Apc*$^{Min/+}$ mice, the cause of death is anemia. Originally and because of bloody stools, it was thought (or assumed) that the severe anemia was due to bleeding neoplasia[11]. In our effort to recapitulate the rapamycin cancer prevention effect in C57BL/6 wild type mice with colon cancer induced by AOM/DSS treatment, we were surprised that control untreated (Eudragit diet) mice with a heavy colon tumor burden and bloody stools had normal

hematocrits. It is curious that AOM/DSS models in this strain lead to mutations in the *Apc*/β-catenin pathway[15], which presumably leads to polyposis as in $Apc^{Min/+}$ mice. This result led us to suspect that the severe anemia observed in $Apc^{Min/+}$ might have an additional etiology due to *Apc* mutations in other organs.

Would chronic rapamycin be a safe and effective deterrent in colon cancer-prone populations? Since it is generally perceived as a potent immunosuppressant used routinely in transplant rejection settings, rapamycin presents a paradox in other settings such as aging and cancer prevention. Several lines of evidence exemplify this puzzle. First, given over a life time, rapamycin unexpectedly for a potent immunosuppressant extends the life and health span of mice as shown by nine repetitions of survival studies by the Intervention Testing Program[7–9]. Second, and unforeseen for an immunosuppressant, rapamycin or rapalogs are also effective in a pre-clinical studies in preventing/delaying cancer in a number of models[33], although as a mono-therapeutic, mTOR inhibitors have had only modest effects[34]. Third, and most unanticipated for a potent immunosuppressant, Mannick et al. showed that everolimus (a rapalog) improved immune function and reduced infections in elders[35,36]. Fourth, Kraig et al. performed a placebo-controlled pilot study (25 healthy older adults, aged 70–95 years), and reported no changes in immune parameters[37]. Thus, for FAP patients and others at risk for colorectal cancer, it appears that an mTOR inhibitor, like rapamycin that targets the large intestine would be worth the risk of manageable adverse effects observed in therapeutic settings[38].

Conclusions:

In a prevention setting, our results indicate that chronic mTORC1 inhibition reduces the incidence of colon neoplasia and significantly extends life span while maintaining a normal hematocrit. We also provide evidence that rapamycin's inhibitory effect on mTORC1 is primarily on cells in colonic crypts via the mTORC1 → S6K → rpS6 and mTORC1 →S6K → eEF2K → eEF2 pathways. Finally, we show that chronic enteric rapamycin has a tumor prevention effect in a related C57BL/6+azoxymethane/DSS (AOM)/DSS) model of large bowel cancer.

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Abbreviations

Apc	adenomatous polyposis coli
FAP	familial adenomatous polyposis
mTOR	mechanistic or mammalian target of rapamycin

mTORC1	complex 1 of mTOR
mTORC2	complex 2 or mTOR
DSS	dextran sodium sulfate
AOM	azoxymethane

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Clinical Practice Points:

Germline mutations in *Adenomatous polyposis coli* (*APC*) cause familial adenomatous polyposis or FAP, which, if untreated, leads to colorectal cancer at an early age. Surgical removal of the colon before polyp formation is the current standard of care for FAP patients. While reducing mortality, this approach permanently reduces the quality of life for these patients and leaves them with the prospect of upper gastrointestinal disease. Inhibition of mechanistic target of rapamycin complex 1 (mTORC1) has been shown to reduce polyposis in $Apc^{Min/+}$ mice. Although $Apc^{MIn/+}$ mice rarely develop colon tumors, they can be converted to a colon cancer model by treatment with dextran sodium sulfate. We now show that targeted enteric delivery of rapamycin delays or prevents colon cancer in this model resulting in a significant increase in survival. Since no effective, safe prevention or treatment options are available for FAP patients, our results offer an obvious and exciting new clinical approach for this dread disease. Based on these results, clinical trials in FAP patients using our novel enteric formulation of rapamycin are underway.

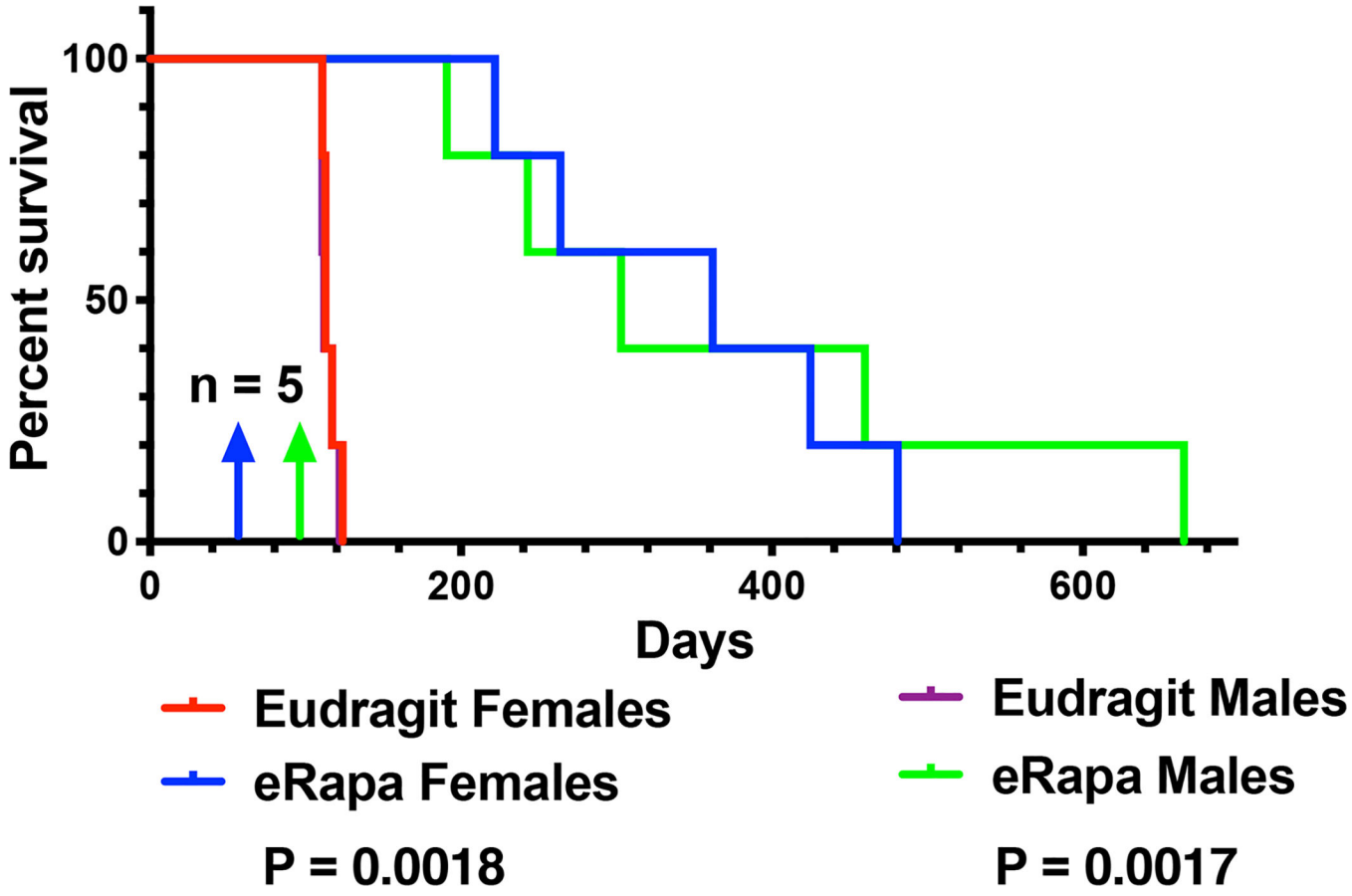

Fig. 1. eRapa effects on life span of DSS-treated *Apc*$^{Min/+}$ female and male mice.

Blue arrow indicates start of eRapa 42 ppm and control diets at 4 weeks while green arrow indicates start of one-week DSS treatment at 8 weeks of age. Life span for females and males was analyzed by the Log-rank (Mantel-Cox) test. P values are indicated below the graphs. ED = Control fed *Apc*$^{Min/+}$ treated with 2.5% DSS and RD = eRapa fed *Apc*$^{Min/+}$ treated with DSS. Note that ED female and male lines (red and purple, respectively) overlap.

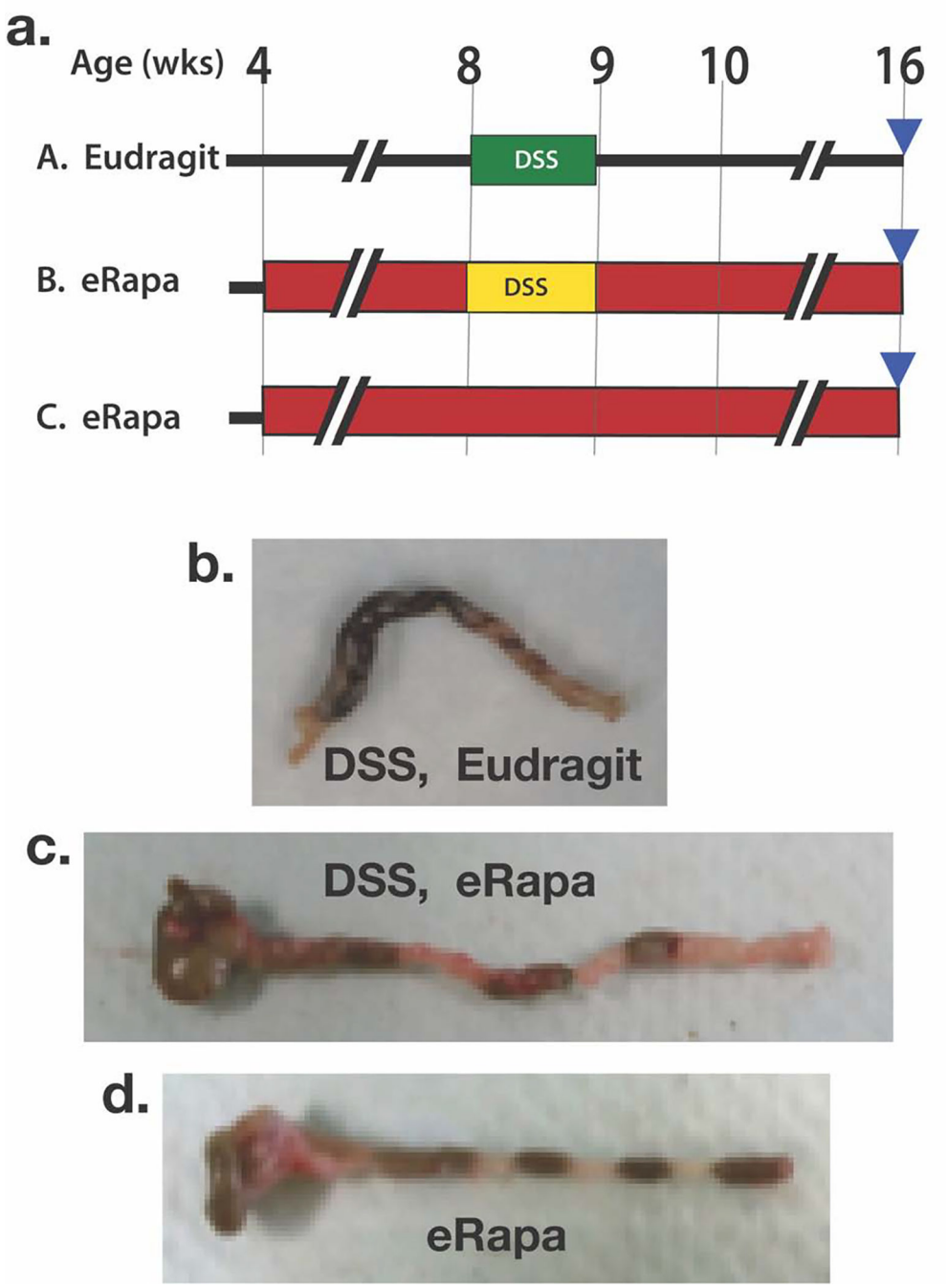

Fig. 2. eRapa effects on colon carcinogenesis in eRapa and control *Apc*$^{Min/+}$ mice.

a) Experimental design of cross section experiments. We started eRapa (red rectangles). and control diets at 4 weeks of age. DSS treatments are indicated by green and yellow boxes. Blue arrowheads indicate when we sacrificed animals for organ collection at 16 weeks. **b)** picture of dissected representative colon from DSS-treated *Apc*$^{Min/+}$ mice fed control diet starting at 4 weeks of age illustrating too many or too large tumors for accurate counts. **c)** Similar image of large bowel from DSS treated *Apc*$^{Min/+}$ mouse fed eRapa diet starting at 4 weeks of age illustrating absence of tumors. **d)** Colon from eRapa treated animal (no DSS).

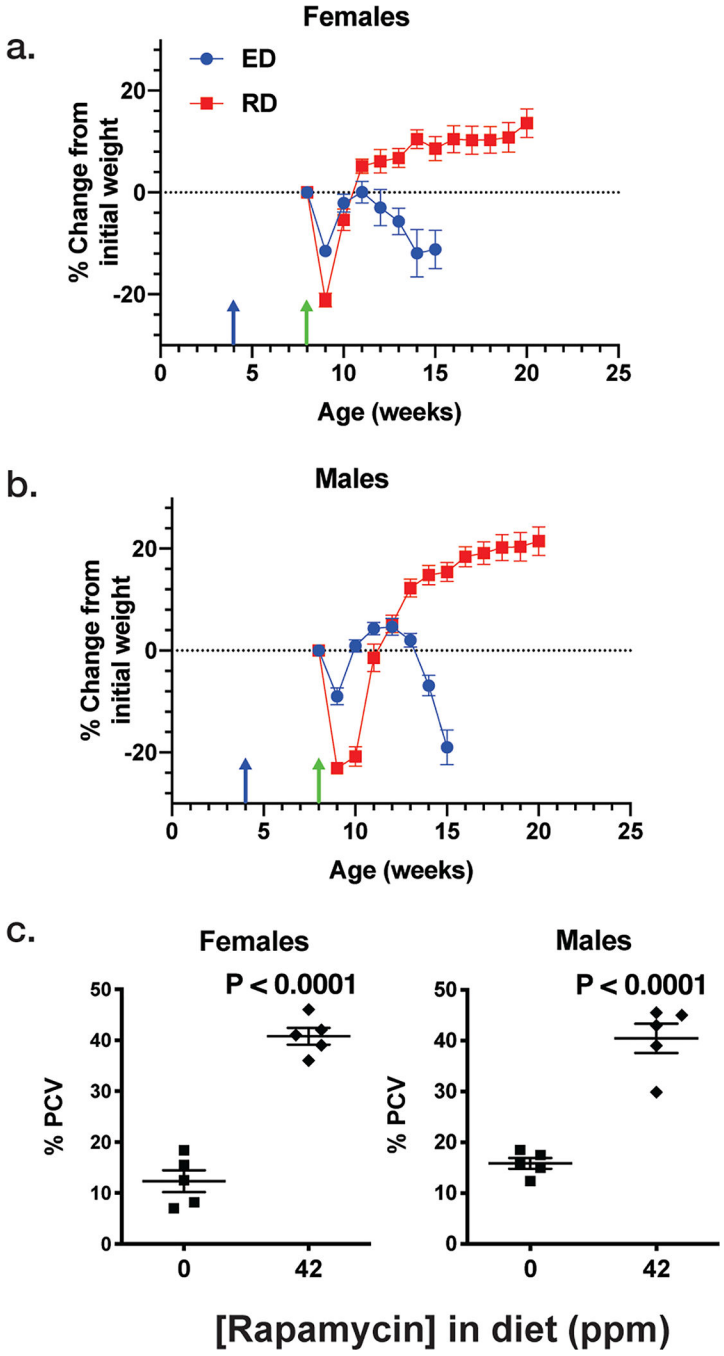

Fig. 3. eRapa effects on weight and hematocrits in DSS treated *Apc*$^{Min/+}$ mice.

(a and b) Female and male, respectively, percent change in weights (y-axis) plotted against age (x-axis). Blue arrows indicate start of eRapa/control diets which continued the rest of their lifetime. Green arrows indicate the start of DSS water for one week. Treatment groups are indicated in the legend of panel a. **c)** Hematocrits (% packed cell volume, PCV) for DSS-treated females and males.

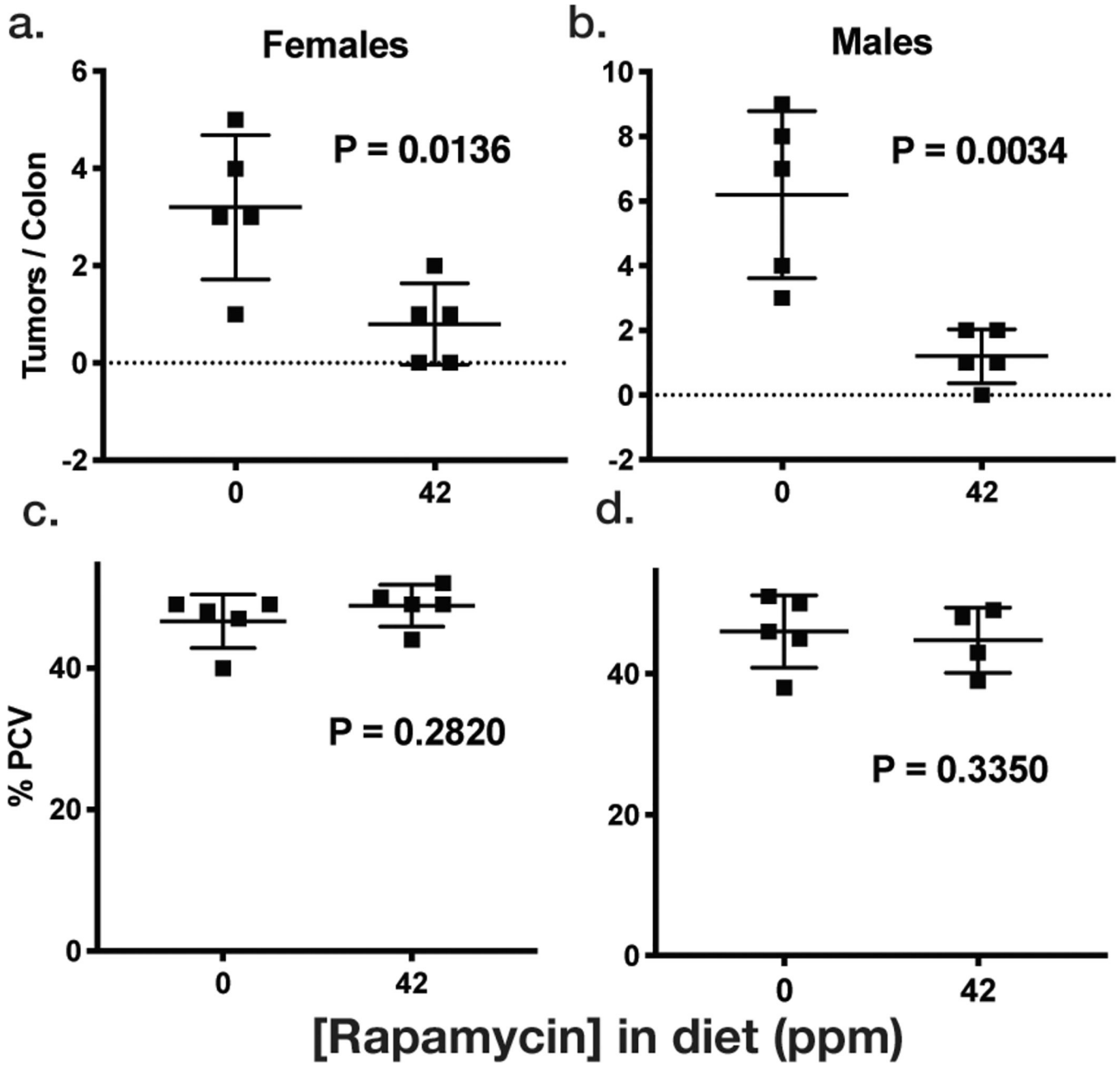

Fig. 4. mTORC1 inhibition delays and/or reduces AOM/DSS colon cancer in C57BL/6 mice.
See methods for AOM/DSS procedure. (**a and b**) Tumors/colon, females and males, respectively. (**c and d**) Hematocrits (%PCV) for females and males, respectively.

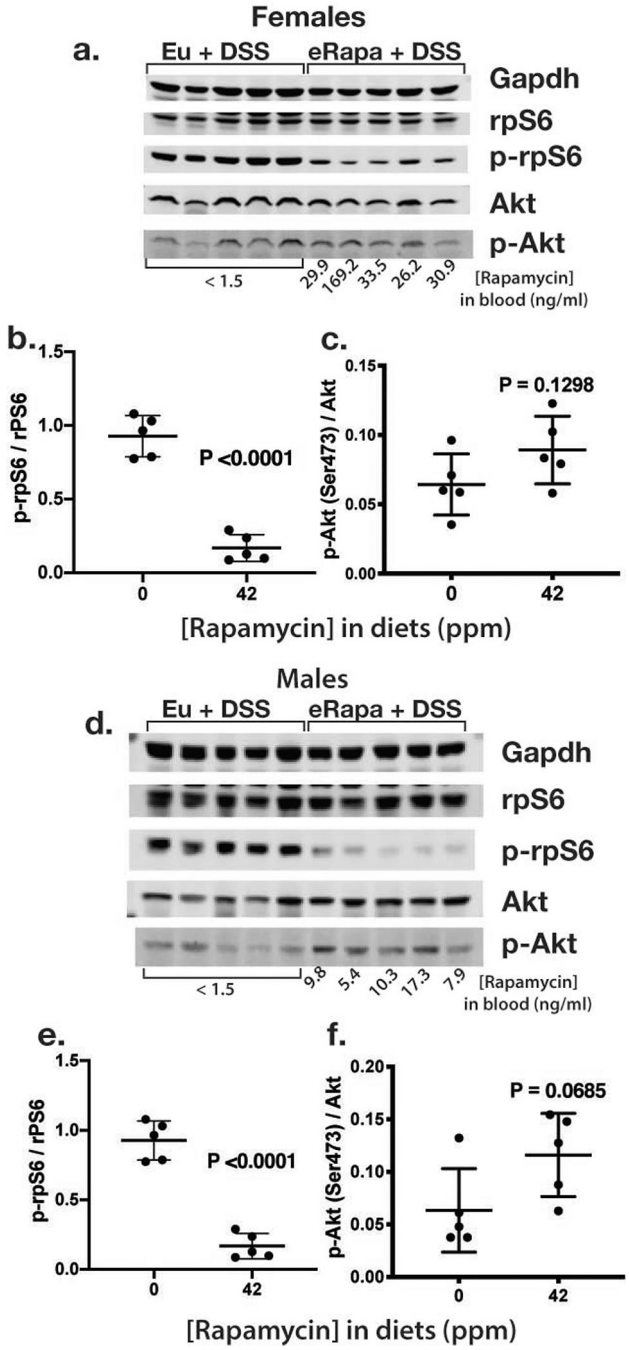

Fig. 5. eRapa effects on colon mTOR effectors.

Immunoblot assays using antibodies specific for the targets on the right. (**a-c**) Representative *Apc*$^{Min/+}$ female blot and graphed intensity values; **(d-f)** representative male blot data and graphs. Treatments are shown on top of each group in the blot images. (**b, c and e, f)** Graphed ratios of phosphorylated Ser 240/244 rpS6 (p-rpS6) to rpS6 and phosphorylated Ser 473 p-Akt (p-Akt) to Akt, respectively. P values are shown in each graph. Blood levels of rapamycin (ng/ml) are shown below each blot image.

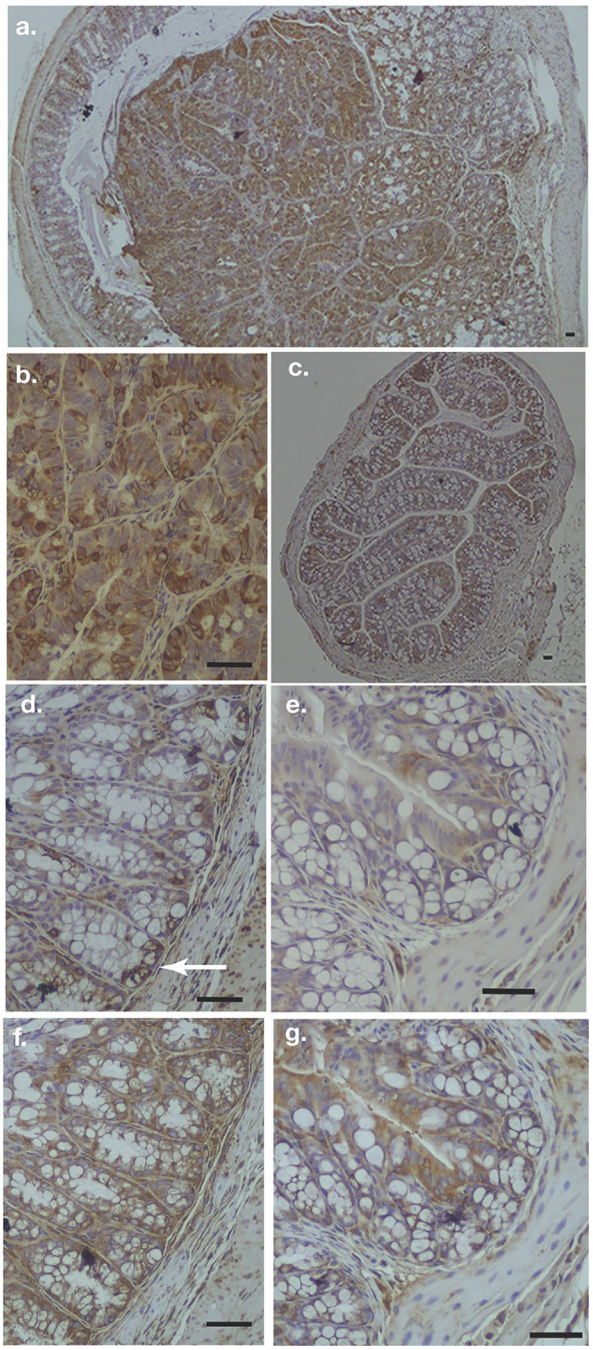

Fig. 6. eRapa effects on rpS6 in *Apc*$^{Min/+}$ colon.

Panels **a-e** are sections immunostained with an rpS6 Ser 240/244 phosphorylation-dependent antibody. Panels **f** and **g** are sections immunostained with a phosphorylation-independent antibody specific for rpS6 (for total protein). (**a**) DSS, Eudragit treated showing large mass in the middle of the colon. (**b**) Magnified sector of tumor in (a) showing prominent phosphorylation of rpS6. (**c**) DSS, eRapa treated colon showing lower rpS6 phosphorylation. (**d**) Magnified view of crypts from (a) indicating rpS6 phosphorylation present in the crypt area (arrow). (**e**) Magnified view of (c) showing undetected rpS6 phosphorylation signal in

crypt areas. (**f and g**) representative sections immunostained for total rpS6 Magnification bars are 50μm.

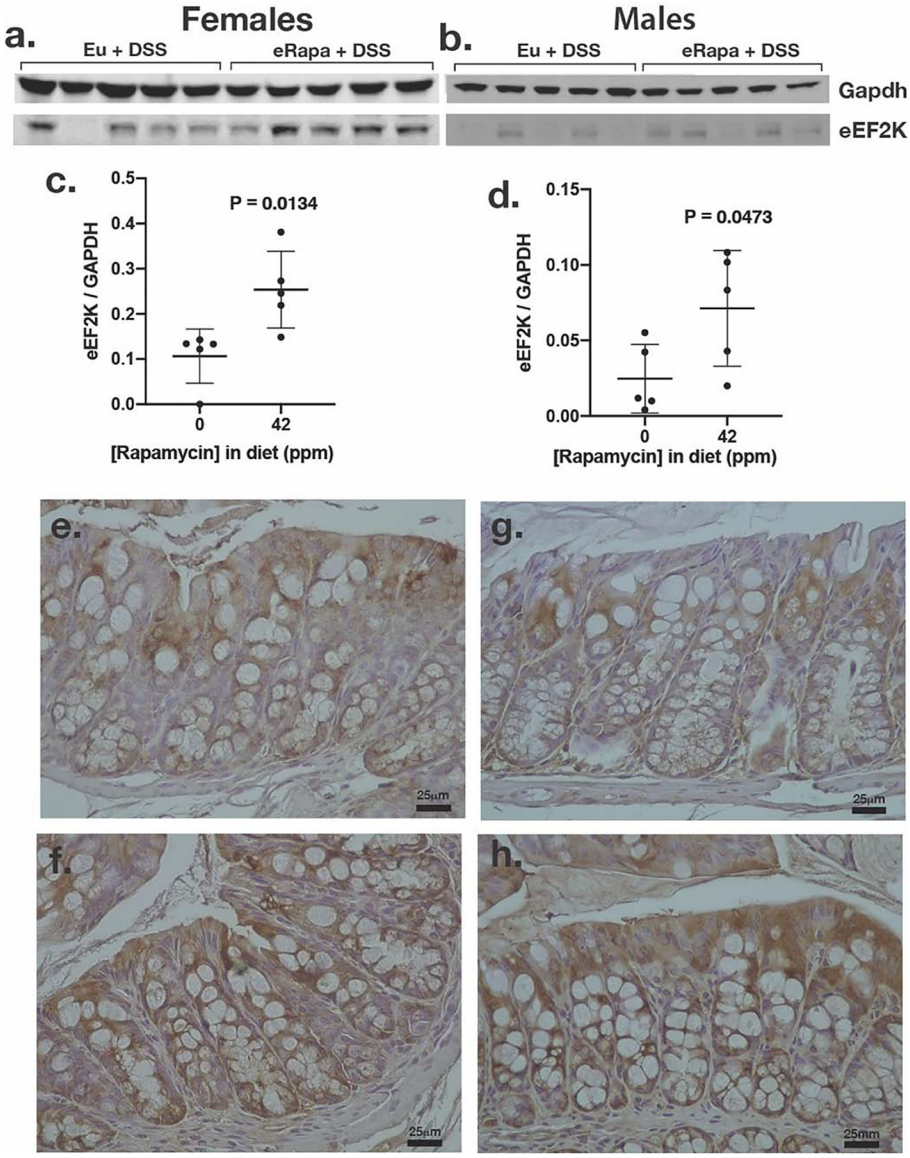

Fig 7. eRapa effects on eEF2K and eEF2 in *Apc*$^{Min/+}$ colon.

Representative immunoblot assays (**a and b**) for female and male, respectively, colon using antibodies specific for the proteins shown on the right. Control (Eu) and 42 ppm rapamycin diets (eRapa) are indicated above the images. Graphed ratios for each sex are shown below the blot images **(c and d)**. P values are shown in each graph. The blood levels of rapamycin in this blot are the same as shown in Fig 5. Panels **e-g** are sections immunostained with eEF2 Thr 56 phosphorylation-dependent antibody for female (**e-f**) and male (**g-h**) colon. DSS Eudragit treated colon are shown in **e and g**, while eRapa treated are shown in **f and h**. Magnification bars are 25 μm.

Table 1.

Colon Tumors (Mean ± SE)

	Eu	Eu +DSS	eRapa	eRapa + DSS
Females	0.8 ± 0.58	*TNC*	0	4.6 ± 1.50
Males	1 ± 0.63	*TNC*	0	4 ± 1.51

TNC = too numerous to count.