

Sex-dependent lifespan extension of *Apc*^{Min/+} FAP mice by chronic mTOR inhibition

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Abstract

Background: *Apc*^{Min/+} mice model familial adenomatous polyposis (FAP), a disease that causes numerous colon polyps leading to colorectal cancer. We previously showed that chronic treatment of *Apc*^{Min/+} females with the anti-aging drug, rapamycin, restored a normal lifespan through reduced polyposis and anemia prevention. Lifespan extension by chronic rapamycin in wildtype UM-HET3 mice is sex-dependent with females gaining the most benefit. Whether *Apc*^{Min/+} mice have a similar sex-dependent response to chronic mTOR inhibition is not known.

Methods: To address this knowledge gap and gain deeper insight into how chronic mTOR inhibition prevents intestinal polyposis, we compared male and female *Apc*^{Min/+} mice responses to chronic treatment with a rapamycin-containing diet. Animals were fed a diet containing either 42 ppm microencapsulate rapamycin or empty capsules, one group was used to determine lifespan and a second group with similar treatment was harvested at 16 weeks of age for cross-sectional studies.

Results: We found that the survival of males is greater than females in this setting ($P < 0.0197$). To explore the potential basis for this difference we analyzed factors affected by chronic rapamycin. Immunoblot assays showed that males and females exhibited approximately the same level of mTORC1 inhibition using phosphorylation of ribosomal protein S6 (rpS6) as an indirect measure. Immunohistochemistry assays of rpS6 phosphorylation showed that rapamycin reduction of mTORC1 activity was on the same level, with the most prominent difference being in intestinal crypt Paneth cells in both sexes. Chronic rapamycin also reduced crypt depths in both male and female *Apc*^{Min/+} mice ($P < 0.0001$), consistent with reduced crypt epithelial cell proliferation. Finally, chronic rapamycin prevented anemia equally in males and females.

Conclusions: In males and females, these findings link rapamycin-mediated intestinal polyposis prevention with mTORC1 inhibition in Paneth cells and concomitant reduced epithelial cell proliferation.

Keywords: Rapamycin, small intestine, polyposis, mTORC1, Paneth cells, crypt stem cells

Introduction

Adenomatous polyposis coli (APC), a tumor suppressor

gene, encodes an inhibitor of the canonical Wnt- β -catenin pathway. APC mutations in the germline cause familial adenomatous polyposis (FAP) [1-3], which, if untreated, leads to colorectal cancer in humans at an early age. Somatic defects in APC function and Wnt signaling are also observed in a majority of colorectal adenomas and carcinomas [4]. Currently, the standard of care for FAP patients is colectomy before the polyps develop [5]. Although this strategy reduces mortality, it significantly deteriorates the quality of life [6]. Hence, there is a clear need to develop better preventative strategies for patients with this class of

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intestinal cancer.

The *Apc^{Min/+}* mouse, an established model to study FAP, presents with multiple adenomas in the intestine, intestinal bleeding, severe anemia, and early death [7]. Previously we showed that the mTOR (mammalian or mechanistic target of rapamycin) inhibitor rapamycin in a targeted enteric release formulation (eRapa) reduced the number of adenomas in the small intestine of female *Apc^{Min/+}* mice leading to a five-fold extension in their mean survival [8]. In addition to the reduction in the number of polyps, eRapa also restored life-long normal hematocrits. Although inhibition of mTORC1 has a role in the reduction of polyposis in *Apc^{fl/fl}* mice [9], the exact mechanism underlying rapamycin effects on adenomas in *Apc^{Min/+}* mice is unknown.

We previously studied female *Apc^{Min/+}* mice since rapamycin trials by the ITP showed a stronger response for life span extensions in females [10]. In humans, daily aspirin administration for more than five years prevented distant metastasis and reduced deaths due to colorectal cancers. Although aspirin also inhibits mTOR [11-13], it was found to only increase the lifespan of male mice in the genetically heterogeneous UM-HET3 strain [14]. How male *Apc^{Min/+}* will respond to chronic mTOR inhibition is an important unknown.

Intestinal polyps in *Apc^{fl/fl}* mice are believed to originate from the *Lgr5⁺* stem cells of the crypts [15]. The self-renewal of stem cells is mediated by mTORC1, which is sensitive to both rapamycin and caloric restriction [16]. To maintain homeostasis in the intestinal crypts, these cells form a niche with the neighboring Paneth cells that are interspersed between the stem cells [17, 18]. Paneth cells not only secrete bactericides to protect the intestinal cell lining but also regulate stem cell function by niche signaling. In wild-type mice, Paneth cells respond to rapamycin treatment as measured by a reduction in phosphorylation of ribosomal protein S6 (rpS6) [16].

A twofold purpose of our study was to compare survival and polyposis preventive effects of chronic rapamycin in *Apc^{Min/+}* male and female mice and to obtain additional insights into its mechanism of action in tumor prevention by an anti-aging drug. Surprisingly, our data show that chronic rapamycin improves the lifespan of *Apc^{Min/+}* males more than in females. Our results also suggest that rapamycin prevents tumors in this model by suppressing mTORC1 activity in the Paneth cells to a similar extent in both sexes leading to a reduction in intestinal crypt length. Additionally, chronic rapamycin prevents anemia in *Apc^{Min/+}* comparably in both sexes.

Methods

Mouse husbandry and diets

We treated and used animals according to Institutional Animal Care and Use Committee and NIH guidelines. We ad libitum fed male and female *Apc^{Min/+}* mice (purchased from Jackson Laboratories Stock No. 002020) our 42 ppm

microencapsulated rapamycin or empty Eudragit capsule (control) diets. Diets were started on four-week-old mice. For longevity experiments, they were allowed to live out their lifespans and euthanasia was performed only on those mice that could not eat or drink or were unable to respond to gentle prodding. The second set of similarly treated animals was sacrificed at 16 weeks of age for cross-sectional studies, and tissue and blood were harvested. We used a 16-week time point for this study based on our previous observations of polyposis in the *Apc^{Min/+}* mice. This harvested tissue was used for immunoblots and histological analysis. Blood concentration of rapamycin was determined as previously described [19].

Hematocrit measurement

We collected approximately 75 μ L of whole blood into a heparinized micro-hematocrit capillary tube (Fisherbrand cat. 22-362-566) by submandibular bleed during tissue harvest. The capillary tubes were centrifuged to pack the cells in the blood and the percentage of packed cell volume (PCV) was measured.

Immunoblots

We performed these assays as previously described [20].

Immunohistochemistry

Harvested tissue was fixed in 10% buffered formalin and embedded in paraffin for sectioning. The sections were heated at 95-100 °C immersed in antigen retrieval buffer containing 10mM sodium citrate and 0.05% tween 20 (pH adjusted to 6.0). Endogenous peroxidases were inhibited by hydrogen peroxide and the sections were blocked using 5% normal goat serum for 1 hour. Tissue sections were then incubated overnight with the primary antibodies at 4 °C. For colorimetric assays, the Signal Stain Boost IHC Detection reagent and Signal Stain DAB Substrate Kit (Cell Signaling Technology, CST 8059) were used for detecting the IHC signal. DNA was stained by hematoxylin counterstain (CST 14166) and an Echo Revolve microscope or a Nikon Eclipse 80i microscope was used to take pictures. Antibodies used were rabbit anti-rps6 (1:600; CST 2217) and rabbit anti-phospho-rps6 ser240/244 (1:2000; CST 5364). For immunofluorescence assays, the sections were incubated with secondary antibodies for 2 hours at room temperature in a dark, humidified chamber and mounted using DAPI medium (Vectashield H-1200). Antibodies used were: goat anti-cKit (1:50; R&D AF1356), donkey anti-goat Alexa 594 (1:500, Invitrogen A32758), goat anti-rabbit Alexa 488 (1:500, Invitrogen A11034).

For measuring the crypt lengths, sections were stained with H&E and the lengths of 40-50 crypts were measured from at least three mice/group from images taken on an Echo Revolve microscope.

Statistical analysis

Lifespan data for the groups was analyzed using the Log-rank (Mantel-Cox) test. The polyp counts and %PCV

were compared using a one-way ANOVA with Tukey's multiple comparisons. All western blot data and the crypts lengths were compared using the Student's *t*-test. A *P*-value of <0.05 was considered significant.

Results

Lifespan: Chronic eRapa increases *Apc*^{Min/+} male survival longer than females

Chronic rapamycin treatment has previously been shown to improve the survival of *Apc*^{Min/+} females [8]. Survival results showed that eRapa extended the lifespans of both males and females compared to controls (Figure 1, *P* < 0.0001 for both sexes, *n* = 5). There was no difference in the survival of Eudragit-treated (control) male and female *Apc*^{Min/+} animals. Interestingly, our analysis showed that *Apc*^{Min/+} males chronically treated with eRapa had a significantly longer lifespan than females (Figure 1, *P* = 0.0197).

Polyp number and hematocrits: eRapa reduced polyp numbers and restored hematocrits similarly in both sexes

Since chronic eRapa had a sexually dimorphic effect on longevity in *Apc*^{Min/+} mice, we ask if there was a sex difference in rapamycin prevention of polyposis. For this purpose, we treated 5 males and 5 females with 0 or 42 ppm eRapa diets (Figure 2A). At 16 weeks of age, we sacrificed the animals and counted the number of polyps in the small intestine. Chronic eRapa significantly reduced the number of polyps in both females (*P* = 0.0004) and males (*P* < 0.0001) to an equal level (*P* = 0.999), Figure 2B. Polyp reduction being close to the same in both males and females treated with eRapa, it is unlikely to account for the longer lifespan in males.

Since *Apc*^{Min/+} mice primarily die from anemia, which chronic eRapa prevents in females [8], we next asked if a sex difference in hematocrit response by rapamycin could account for the difference in longevity effects.

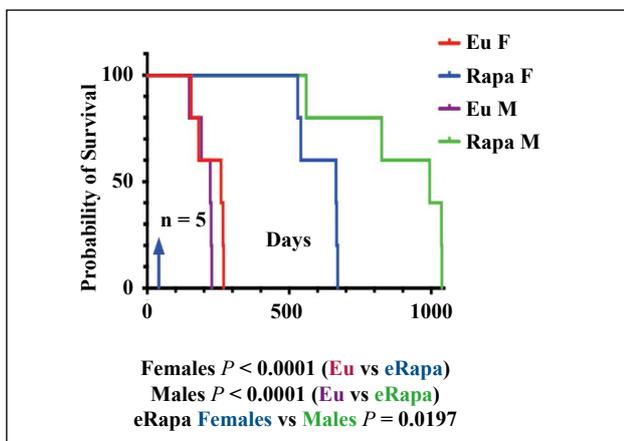


Figure 1. Chronic rapamycin extends longevity of male *Apc*^{Min/+} mice greater than females. Blue arrow indicates the start of eRapa 42 ppm and control (Eudragit) diets at 4 weeks of age. Lifespan statistics for the groups were calculated using the Log-rank (Mantel-Cox) test. *P* values are below the graph.

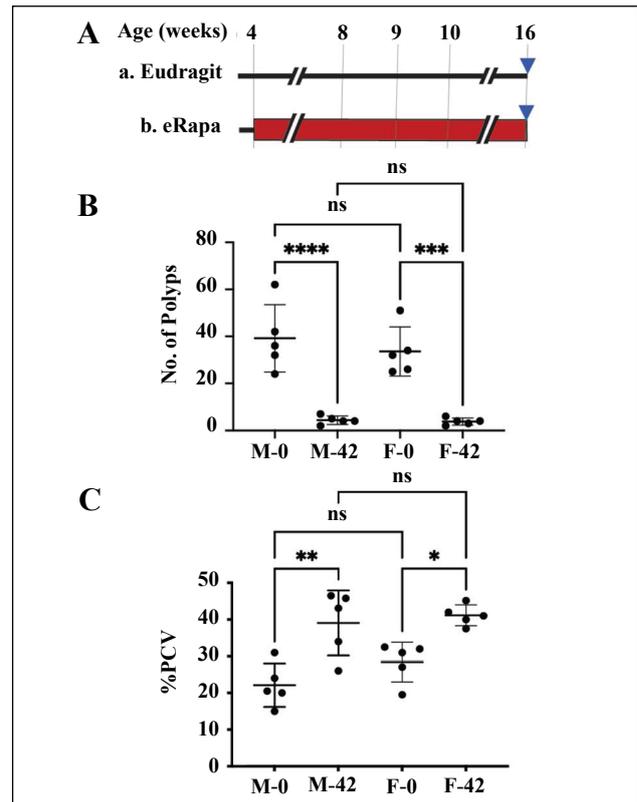


Figure 2. Rapamycin reduces small intestine polyposis in female and male *Apc*^{Min/+} mice. (A) Experimental design for cross-section experiments. Red rectangle indicates rapamycin treatment period. Blue triangles indicate the age at which we collected tissues. (B) Graph of the number of polyps for females (F) and males (M) on the indicated diets (0 or 42 ppm eRapa). (C) Rapamycin prevents anemia in female and male *Apc*^{Min/+} mice. PCV = packed cell volume. Graph showing packed cell volume (PCV) percentages for females and males on the indicated diets. * *P* ≤ 0.05, ** *P* ≤ 0.01, *** *P* ≤ 0.001, **** *P* ≤ 0.0001

At tissue harvest, the percentage of packed cell volume (%PCV) of the eRapa treated *Apc*^{Min/+} mice (both sexes) was significantly improved (*P* = 0.0024 for males and *P* = 0.0219 for females), with no difference between the male and the female eRapa treated animals (*P* = 0.95), Figure 2C. These data show that reduction in tumor number and anemia amelioration are mostly responsible for the extension in the lifespan of rapamycin-treated male and female *Apc*^{Min/+} mice. However, they do not provide clues as to why chronic inhibition of mTOR results in longer-lived *Apc*^{Min/+} males compared to females (Figure 1). We next investigated mTORC1 status for potential changes in response to eRapa treatment.

Status of mTORC1. Chronic eRapa decreases mTORC1 activity in small intestine tissue lysates

Previously, it has been documented that chronic rapamycin brings about a reduction of mTORC1 activity in C57BL/6 female small intestine [8]. To address the question of mTORC1 status in *Apc*^{Min/+} males and females, we used immunoblot assays of small intestine lysates in cross-section experiments. As expected, chronic treatment of male *Apc*^{Min/+} fed 42 ppm eRapa diet resulted in a reduction of phosphorylation.

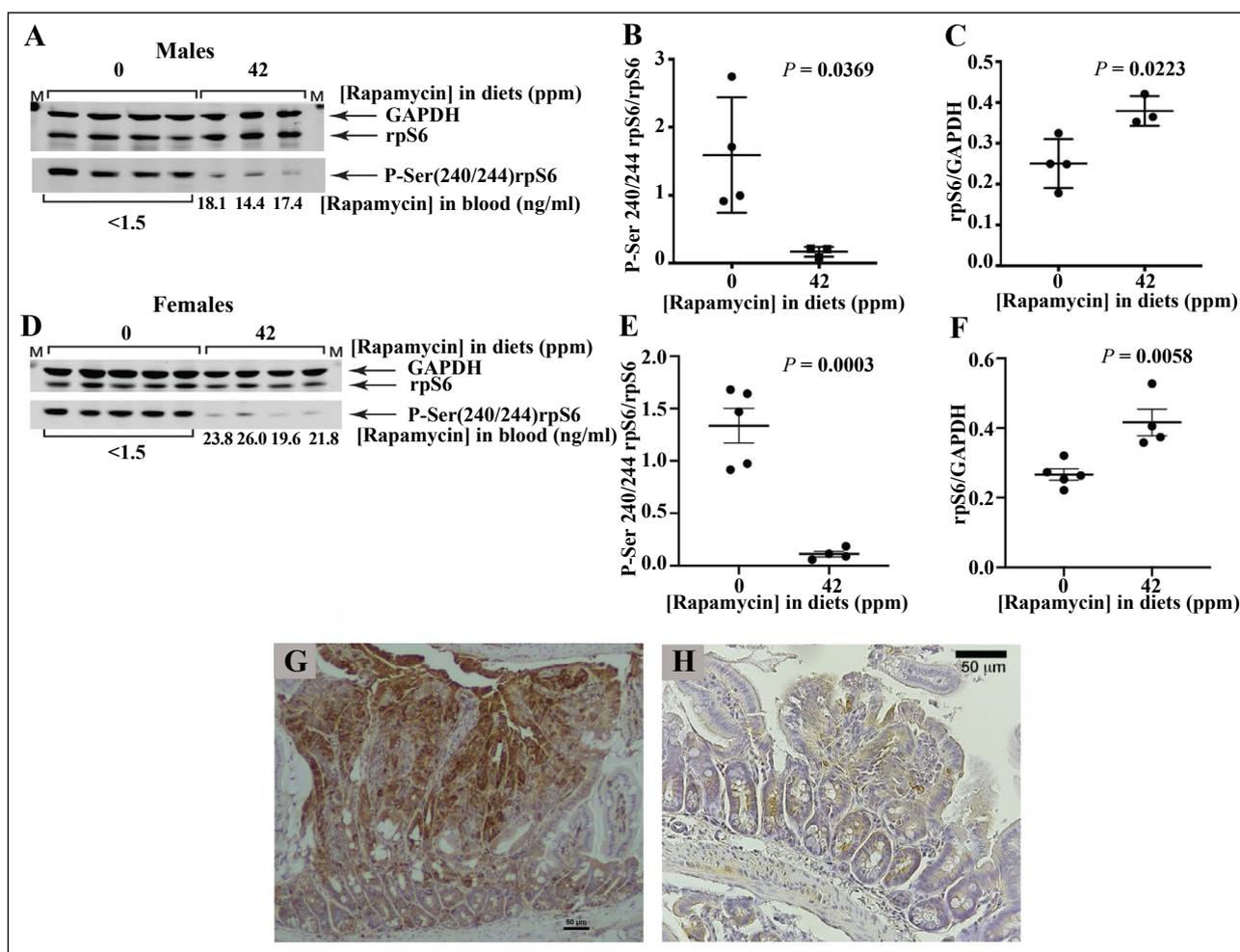


Figure 3. eRapa effects on mTORC1. Representative immunoblot assay of small intestine lysates prepared from *Apc^{Min/+}* male (A) and female (D) mice respectively. Diets indicated above the blots, antibodies to the right of each image, and rapamycin concentrations in blood are below the blots. Graphs show the quantification of the immunoblot data as measured by the ratio of the intensity values for phosphorylation state-dependent signal (P-Ser 240/244 rpS6) to phosphorylation state-independent (rpS6) signal (B, E) and rpS6 intensity values relative to GAPDH (C, F). Phosphorylated rpS6 expression in small intestine of control (G) and eRapa treated (H) mice.

(Ser240/244)-dependent intensity values relative to phosphorylation state-independent intensity (total rpS6 protein) in the small intestine lysates of males show a reduction with rapamycin treatment (Figure 3A and 3B). Rapamycin treatment also raised the levels of rpS6 signal relative to GAPDH (Figure 3C), a response not observed in C57BL/6 intestine [8] or colon [20]. Immunoblot assays of female small intestine lysates also demonstrate a similar reduction of rpS6 phosphorylation (Figure 3D and 3E) and an increase in rpS6 protein signals (Figure 3F). As determined by immunohistochemistry, polyps in the small intestine of control animals (Figure 3G) showed a markedly higher rpS6 phosphorylation which was absent in the small polyp in the eRapa group (Figure 3H). These data indicate mTORC1 inhibition in the small intestine in both sexes in response to rapamycin treatment.

mTORC1 Crypt Status. Chronic eRapa reduces mTORC1 activity in Paneth cells in *Apc^{Min/+}* mice

Yilmaz *et al.* [16] linked a calorie restriction-associated increase in the renewal of the small intestine crypt stem cells in C57BL/6 mice with the repression of mTORC1

in Paneth cells. We asked: what effect would chronic eRapa diets have on mTORC1 status in *Apc^{Min/+}* Paneth cells? This is an important question for two reasons: 1) Paneth cells constitute a niche for intestinal crypt cells [21]; 2) Paneth cell mTORC1 plays a critical role in nutrient and rapamycin responses for stem cell renewal in the niche [16]. In both sexes, immunohistochemistry using an antibody specific for phosphorylation of Ser-240/244 in rpS6 demonstrated eRapa-mediated suppression of staining prominently in crypt cells (Figure 4, females and Figure 5, males). Thus, at the cellular level, there was no discernable sex difference in chronic rapamycin-mediated mTORC1 suppression.

We postulated that chronic rapamycin reduced rpS6 phosphorylation (and mTORC1 activity) in Paneth cells. As a test, we used an antibody specific for cKit in immunofluorescence assays of small intestine tissue sections. cKit receptor tyrosine kinase and its ligand, stem cell factor (SCF), are known to play important roles in various mammalian organs through several signaling pathways including PI3 kinase [22]. It is also known to be specifically expressed in intestinal crypt Paneth cells [23]. Supporting

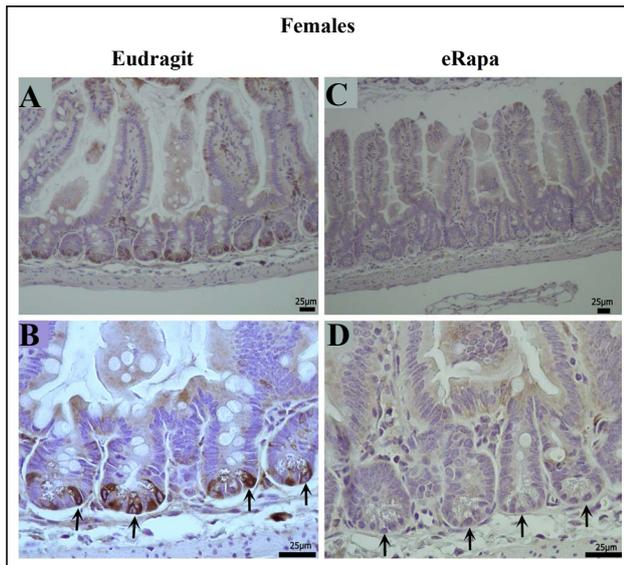


Figure 4. Chronic eRapa represses mTORC1 activity in female intestinal crypt niche primarily in Paneth cells. (A, B). Intestinal preparations from Eudragit fed females. Arrows in panel **B** point to prominent staining of crypt cells with an antibody specific for P-Ser(240/244)-rpS6. In eRapa fed specimens (**C, D**), no signal is detected in the crypt niches (arrows in panel **D**). Panels **A** and **C** show lower magnification of villi and crypts in mice fed Eudragit and eRapa diets respectively.

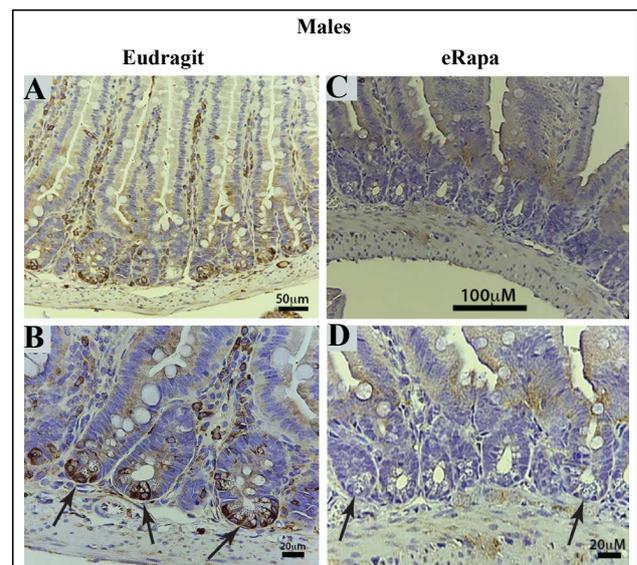


Figure 5. Chronic eRapa represses mTORC1 activity in male intestinal crypt niche primarily in Paneth cells. (A, B). Intestinal preparations from Eudragit fed males. Arrows in panel **B** point to prominent staining of crypt cells with an antibody specific for P-Ser(240/244)-rpS6. In eRapa fed specimens (**C, D**), no signal is detected in the crypt niches (arrows in panel **D**). Panels **A** and **C** show lower magnification of villi and crypts in mice fed Eudragit and eRapa diets respectively.

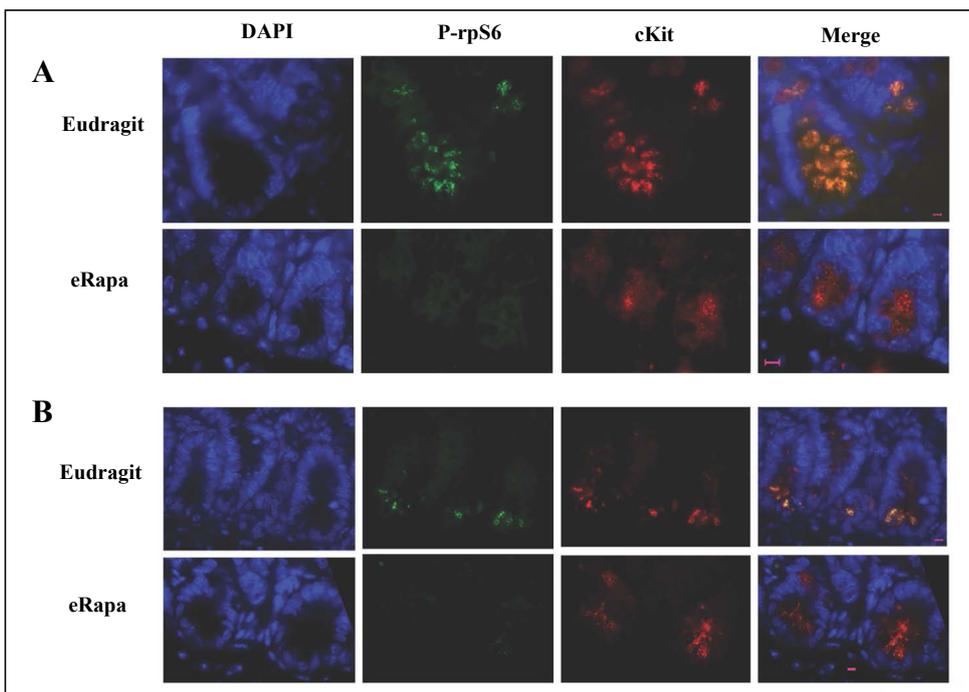


Figure 6. Co-localization of cKit and reduced phosphorylated rpS6 by chronic rapamycin in intestinal crypts. Panels **A** and **B** show results using tissue sections from females and males respectively, which were prepared from mice fed eRapa diets (42 ppm rapamycin) or Eudragit controls. DAPI identifies nuclei (blue). Antibodies specific for P-Ser(240/244)rpS6 are green and those specific for cKit are red. Magnification bars are 5 μ m.

our postulate, a representative panel of microscopic images shows co-localization of fluorescence generated by antibodies specific for P-Ser(240/244)rpS6 and cKit in [Figure 6A](#), females, and [Figure 6B](#), males. Consistent with previous assays for mTORC1 responses to chronic rapamycin, we could not detect any sex difference in Paneth cells.

eEF2 Kinase: Evidence that chronic eRapa reduced

translation elongation

In a liver regeneration setting, rapamycin preferentially inhibits S6 kinase 1(S6K1) over 4E-BP1 [24] suggesting that the mTORC1-4E-BP1 pathway might not be a limiting pathway in polyp promotion. Translation initiation control by mTORC1 has been extensively studied, while mTOR's effect on translation elongation has gotten less scrutiny. Eukaryotic elongation factor 2 kinase (eEF2K)

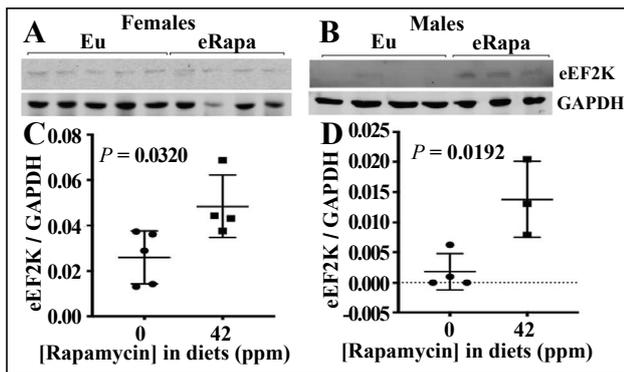


Figure 7. eRapa effects on eukaryotic elongation factor 2 kinase (eEF2K). Representative immunoblot assay of small intestine lysates prepared from *Apc^{Min/+}* female (A) and male (B) mice respectively. Diets indicated above the blots, antibodies to the right of the lanes. Graphs showing the quantification of the immunoblot data as measured by the ratio of the intensity values for eEF2K signal relative to GAPDH signal (C, D).

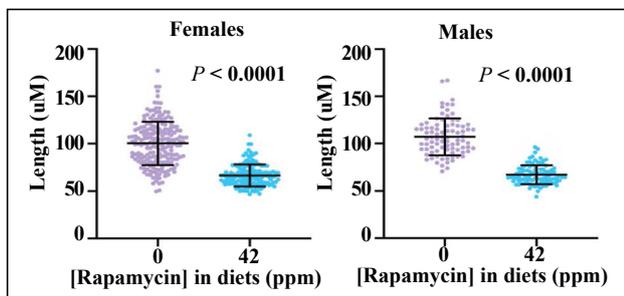


Figure 8. Rapamycin reduced crypt depths in female and male intestinal crypt measurements.

is a key node in the regulation of translation elongation. In anabolic settings, S6K1 phosphorylates eEF2K to inhibit its activity [25]. In conditions where mTORC1 (and S6K1) are inhibited, dephosphorylation of these residues frees eEF2K to phosphorylate eEF2, which slows elongation of translation [25]. Relevant to our study, Faller et al. showed that translation elongation by way of S6K1 is a significant factor for tumor growth in *Apc^{fl/fl}* mice in addition to mTORC1 effects on protein synthesis [26]. To explore the effects of chronic eRapa on this axis, we used an immunoblot assay of eEF2K levels. In both males and females, rapamycin increased the levels of eEF2K significantly in small intestine lysates (Figure 7 A-D).

Crypt Lengths: Chronic eRapa reduced intestinal crypt lengths

Faller et al. reported that rapamycin and cycloheximide reduced intestinal crypt size in *Apc^{fl/fl}* mice [26]. Would this be the case for rapamycin-treated *Apc^{Min/+}* mice? Compared to *Apc^{Min/+}* mice fed Eudragit diets, both female and male mice on rapamycin diets had significantly reduced intestinal crypt depths ($P < 0.0001$), Figure 8.

Discussion

Our experiments to determine if there were sex differences

in response to chronic rapamycin in *Apc^{Min/+}* mice revealed an unexpected and important difference; males had greater survival benefits than females. Our other assessments of mTORC1 effects in each sex revealed no other detectable differences. In earlier rapamycin trials by the Intervention Testing Program (ITP), UM-HET3 females fared significantly better than males in a dose-dependent manner [10]. If cancer prevention played a role in longevity extension by chronic rapamycin treatments in this setting, the ITP results would have predicted outcomes opposite to what we found. Flurkey et al. [27] reported no difference in a lifespan extension of UM-HET3 females and males by diet restriction, which reduces the activity of mTORC1, although in a circadian-dependent manner [28]. Combined, these data point to a significant difference in how rapamycin extends lifespan compared to diet restriction, and also different in cancer-prone models. For example, Livi et al. [29] found no interaction of sex with longevity extension by chronic rapamycin in the *Rbl^{+/-}* cancer-prone model.

Why chronic eRapa works better in males in the *Apc^{Min/+}* cancer-prone model is a mystery. Solving this mystery will require additional studies perhaps focusing on Wnt/ β -catenin signaling in other organs such as adipose. Curiously, it has been proposed that rapamycin feminizes males [30, 31] as a possible reason for the sexual dimorphism in its effect on longevity. Again, we cite Livi et al.'s rapamycin *Rbl^{+/-}* report [29] and Flurkey et al.'s UM-HET3 diet restriction study [27] showing no sex differences as counterarguments. Thus, the interaction of sex in the rapamycin longevity effect, like diet restriction, probably depends on the experimental setting including mouse genotype.

This is the second demonstration that delivery of rapamycin to the location of polyp formation in the small intestine is an effective strategy for the prevention of tumor development in females, and now for the first time in males. To gain an initial understanding of how chronic rapamycin achieves this effect, we used immunoblots and immunolocalization assays to determine the status of a mTORC1 downstream effector. Our data indicate that Paneth cells in the crypts have the most prominent reduction in rpS6 Ser 240/244 phosphorylation. However, what does this mean in terms of the prevention of polyposis? *Lgr5⁺* intestinal crypt stem cells (ICSCs) originate polyps in *Apc^{fl/fl}* mice [15]. Paneth cells are thought to be supporting cells for ICSCs in the crypt niche [21, 32], although were recently found to be dispensable resulting in a remodeled crypt with enteroendocrine and tuft cells supporting *Lgr5⁺* stem cells [33]. Assuming that ICSCs are the cells-of-origin for polyps in *Apc^{Min/+}* mice, what could be the mechanism by which Paneth cells mediate the prevention of polyposis by chronic mTORC1 inhibition? In wild-type mice, Yilmaz et al. provided an important clue by showing that inhibition of mTORC1 in Paneth cells by diet restriction or rapamycin increased intestinal crypt stem cell renewal [16]. Yilmaz et al. attributed this effect to an increase in Bst1, an ectoenzyme that converts NAD⁺ to cyclic ADP ribose

(cADPR). As a paracrine effector, cADPR promotes proliferation (or self-renewal in stem cells) by activating calcium signaling. In the *Ap^{c^{Min/+}}* intestine, our attempts to assay Bst1 in response to chronic rapamycin were inconsistent leaving this possibility open. Importantly, we did observe a reduction in crypt depth by chronic rapamycin suggesting an increase in ICSCs renewal and reduction in transit-amplifying cells of the crypt. Whatever the reason our major finding remains that chronic rapamycin prevents polyposis and extends the health span of an accepted model of FAP.

In addition to S6K1 → rpS6, mTORC1 regulates translation elongation through the S6K1 → eEF2K → eEF2 pathway. In our studies of DSS-induced colon cancer in *Ap^{c^{Min/+}}* mice, we observed that chronic rapamycin resulted in increased levels of eEF2K and elevated levels of Thr 56 phosphorylation of eEF2 in colonic crypts indicating a reduction in elongation and protein synthesis [19]. These data were consistent with the idea that chronic rapamycin prevents tumorigenesis in this setting by a mTORC1 reduction of protein synthesis via two pathways, S6K1 → rpS6, and S6K1 → eEF2K → eEF2. Thus, we expected that chronic rapamycin would have the same effect on the eEF2 pathway in the small intestine of *Ap^{c^{Min/+}}* mice. Indeed, we observed a significant increase in eEF2K levels by immunoblot assays consistent with our expectations. These data suggest that the rpS6 and eEF2 pathways have a combined role in preventing small intestine polyposis. However, there is a caveat to this interpretation since the results of our immunohistochemistry assays of Thr 56 phosphorylation in crypt eEF2 were inconsistent despite repeated attempts under varying conditions. Thus, the effects of chronic rapamycin on translation elongation remain to be fully tested.

Contrary to long-standing beliefs, chronic rapamycin appears to be safe and effective as an anti-cancer or anti-aging intervention. It remains to be determined if localized delivery of rapamycin on polyposis in FAP patients will be as effective as it is in mice. It is clear that minor adverse effects associated with chronic rapamycin compared with the potential benefits strongly suggest that its use would be worth the risk if it works as well in FAP patients (and perhaps other colitis driven diseases in people) as it does in mice. Since eRapa is effective in preclinical trials of wild type mice by the ITP, including those started late in life [34], we suggest that chronic rapamycin would be a good candidate for the prevention of polyposis and other colorectal cancers in post colectomy patients at risk for another duodenal adenomatosis [35], and lastly in older patients who are under surveillance by colonoscopy.

Declarations

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Potential financial conflict of interest: The University of Texas Health Science Center at San Antonio has applied for a patent, U.S. Patent Application No. 13/128,800, by inventors Zelton Dave Sharp, Randy Strong, and Paul Hasty for an encapsulated rapamycin formulation used in this paper. Under a licensing agreement between Emtora Biosciences (formerly Rapamycin Holdings, Inc.) and the University of Texas Health Science Center San Antonio, R. Strong, Z.D. Sharp, P. Hasty, and the University are entitled to milestone payments and royalty on sales of microencapsulated rapamycin. The university has a plan for managing conflicts of interest under its "Policy and Procedures for Promoting Objectivity in Research by Managing, Reducing, or Eliminating Conflicts of Interest."

Conflicts of interest: All authors declared that there are no conflicts of interest

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