

Extracellular calcium-sensing receptor/PTH knockout mice colons have increased Wnt/ β -catenin signaling, reduced non-canonical Wnt signaling, and increased susceptibility to azoxymethane-induced aberrant crypt foci

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Epidemiological evidence suggests increased dietary calcium and dairy products reduce the onset of colon cancer. To understand a role of the colonic extracellular calcium-sensing receptor (CaSR) in calcium-mediated chemoprevention of colon cancer, we induced formation of aberrant crypt foci (ACF) caused by azoxymethane (AOM) injection in 'rescued' CaSR $-$ /PTH $-$ (C $-$ /P $-$) double knockout colons compared with colons from control CaSR $+$ /PTH $+$ (C $+$ /P $+$) mice. C $-$ /P $-$ colonic epithelia had increased Wnt/ β -catenin signaling as evidenced by 3–8-fold increases in Wnt3a, CyclinD1, and MMP-7 proteins compared with C $+$ /P $+$ colonic epithelia. The C $-$ /P $-$ colonic epithelia had reduced Wnt5a and Ror2, and a three-fold increase in TNFR1 compared with C $+$ /P $+$ epithelia. The C $-$ /P $-$ colons and small intestine had extensive neutrophil infiltration with myeloperoxidase (MPO) levels 18-fold higher than C $+$ /P $+$ small intestine and colon. Saline-injected C $-$ /P $-$ colons had the same number of ACF/cm² as C $+$ /P $+$ colons, which were injected with AOM. However, there were eight times more ACF/cm² in the C $-$ /P $-$ injected with AOM compared with C $+$ /P $+$ colons, which received AOM. Together our results suggest both inflammation and Wnt/ β -catenin signaling are increased in the epithelia of 'rescued' CaSR/PTH double knockout colons, and the capacity for non-canonical Wnt signaling through Wnt5a/Ror2 engagement is reduced. The loss of the colonic CaSR increased the number of ACF/cm² in response to AOM injection, suggesting colonic CaSR may mediate the chemoprotective effect of increased dietary calcium against colorectal cancer observed in humans.

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Increases in dietary calcium and dairy products have been demonstrated to reduce the onset of colorectal cancer in humans and rodents.^{1–3} The mechanism by which this chemoprevention occurs is not understood.⁴ Although increased dietary calcium could chelate and reduce bile acid concentration in the lumen, a different target of increased luminal calcium is the extracellular calcium-sensing receptor or CaSR. The CaSR is a G-protein coupled receptor of class C, originally cloned from bovine parathyroid and subsequently found to be highly expressed on the apical membrane of colonic crypt epithelial cells from the mid-crypt to the surface

epithelia.^{5–8} The CaSR is progressively lost during the transition from adenoma to colon cancer in humans,^{7–11} because of epigenetic silencing. Activation of the CaSR on several colonic adenocarcinoma cell lines will inhibit Wnt/ β -catenin signaling.^{10,12} Increasing colonic CaSR expression in colon cancer might allow dietary calcium to regulate colonic Wnt/ β -catenin activity levels.

Studies of CaSR function in adenocarcinoma cell lines have shown that calcium stimulation of the CaSR will inhibit Wnt/ β -catenin signaling by stimulating autocrine production of Wnt5a. This Wnt ligand then mobilizes the ubiquitin

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ligase Siah2, which degrades β -catenin. When wild-type APC was expressed in these cells, autocrine Wnt5a secretion did not occur.¹² However, in normal colonic myofibroblasts, CaSR stimulation by calcium increases the synthesis and secretion of Wnt5a, while on the physically juxtaposed epithelia, CaSR stimulation generates increases in Ror2, the receptor for Wnt5a. This CaSR-mediated paracrine relationship of increased Wnt5a/Ror2 engagement increases CDX2, sucrase-isomaltase,¹³ and villin¹⁴ in normal intestinal epithelial cells. The CaSR-mediated Wnt5a/Ror2 engagement has also been shown to reduce TNFR1 expression and prevent TNF- α stimulated damage to model epithelial cells.¹⁵ Together these studies have suggested calcium stimulation of intestinal and subepithelial CaSR selectively activates Wnt5a and its receptor Ror2, and that both anti-inflammatory and increased epithelial differentiation arise from Wnt5a/Ror2 engagement in the intestine. The requirement of truncated APC to allow CaSR-mediated secretion of Wnt5a from adenocarcinoma cells suggested calcium chemoprevention could occur in spite of increased Wnt/ β -catenin signaling. Notably, the highest rates of survival in stage II of colon cancer are associated with increased Wnt5a protein expression.^{16,17}

There are two types of CaSR knockout mice.^{18,19} When intestinal CaSR is ablated in the floxed mouse, the colonic crypts have been shown to be elongated and hyperproliferative.²⁰ The second type is the global 'rescued' knockout where the perinatal lethality, because of increased PTH secretion, is 'rescued' through mating with PTH heterozygotes to generate a double knockout (CaSR- / PTH-) (C- / P-). These mice that are fertile are sustained by 1.5% calcium in their drinking water.^{18,21,22} The intestinal phenotype of the C- / P- mouse has not been reported.

In the current report, we have characterized increased Wnt/ β -catenin signaling in the colonic crypts of the C- / P- mice and challenged them with the carcinogen azoxymethane (AOM) to generate aberrant crypt foci (ACF). As described herein, we find that both the small intestine and colon of the C- / P- mice had increased neutrophil infiltration, while the colonic epithelia had increased Wnt3a, CyclinD1, and MMP-7 protein expression and substantially reduced Wnt5a and Ror2 expression. Consistent with reduced Wnt5a/Ror2, the C- / P- colons have increased TNFR1 compared with C+ / P+ normal mice. Following AOM injection, the C- / P- colons displayed eight times more ACF/cm² than control mice. Saline-injected C- / P- mice had the same number of ACF/cm² as control C+ / P+ mice, which were injected with AOM. Together our findings suggest 'rescued' C- / P- mice are more sensitive to AOM-induced crypt foci, and provide new data to suggest how increased dietary calcium, by activating the colonic CaSR, could attenuate colon cancer development.

MATERIALS AND METHODS

Mice

Breeding pairs of CaSR+ / PTH+ (C+ / P+), CaSR+ / PTH- (C+ / P-) and CaSR- / PTH- (C- / P-) were

obtained from EM Brown (HMS, Boston, MA, USA). Mice were maintained in standard cages and tap water, which contained 0.7% calcium dihydrate (C+ / P-) and 1.5% calcium dihydrate (C- / P-) *ad libitum* as previously described.^{21,22} All animal procedures were in accordance with a protocol approved by the Animal Care Committee at Queen's University.

AOM Injections and ACF Determinations

To evaluate the effect of AOM three groups (six mice/group) of mice were used. C+ / P+ and C- / P- were used between 8 and 15 weeks of age. Two groups (C+ / P+) and C- / P- were injected intraperitoneally with 15 mg/kg of AOM (Santa Cruz Biochemicals, Santa Cruz, CA, USA) and one group of C- / P- received the same volume of saline ip. After 24 days, mice were anesthetized with Isoflurane, then killed by cervical dislocation. The entire colon was removed and measured, flushed with Tris-buffered saline to remove fecal contents, opened longitudinally from the anus to the cecum, and fixed between filter papers in 10% neutralized formalin overnight at 4 °C. Following the protocol described by Bird,²³⁻²⁶ the fixed colons were placed in buffered saline containing 0.2% methylene blue for 10-12 min. The stained colons were then placed on a glass slide with the luminal side up. By viewing the stained colons with a light microscope at a magnification of $\times 40$, the colons were assessed for ACF. The number of ACF per colon was determined and expressed per length of the individual colon.

At day 3 after AOM injection, two of the C- / P- mice showed inertia, ruffled fur, and > 12% weight loss, and were killed. At day 10, another of the C- / P- mice showed the same clinical symptoms and was killed. Thus, by day 10, 50% of the C- / P- mice had died. No clinical symptoms were noted in the control C+ / P+ mice, which received the same dose of AOM or in the C- / P- mice, which had received saline injections.

Histology

Colon sections were fixed in 10% formalin and embedded in paraffin. Sections of 5 μ M were stained H&E. Photomicrographs were taken using a Nikon Elipse TS100 microscope.

Intestinal Epithelial Cell Isolation

Isolation of epithelial cells from jejunum or colon from C+ / P+ and C- / P- mice was performed as previously described.¹³ A minimum of three mice per group were collected. Cells were collected by centrifugation (1500 g/ 10 min at 4 °C then immediately resuspended in lysis buffer.

Western Blot Analyses

Tissue samples in lysis buffer were sonicated for 3 s, centrifuged, and aliquots denatured at 70 °C for 10 min in sample buffer and separated by SDS-PAGE as described previously.¹²⁻¹⁵ Densitometry was performed using Image J

v1.34 (NIH) and a Personal Densitometer (Molecular Dynamics). Antibody suppliers and dilutions were as follows: Wnt3a (1:1000), CyclinD1 (1:500), MMP-7 (1:1000), and Wnt5a (1:1000) were from R&D systems (Minneapolis, MN, USA). Monoclonal anti- β -actin (1:1000) was purchased from Sigma (St Louis, MO, USA). Anti-Ror2 rabbit polyclonal antibody (1:1000) and anti-TNFR1 (1:1000) were from Cell Signaling Technology (Boston, MA, USA). Horse radish peroxidase conjugated anti-mouse or rabbit antibodies (1:2000) were from Sigma Chemical (Oakville, ON, USA). The enhanced chemiluminescence SuperSignal kit was from Pierce (Rockford, IL, USA). Protease inhibitors were from Boehringer Ingelheim.

MPO Activity Measurements

In all, 1 cm² sections of either jejunum or mid-colon from C+/P+ and C-/P- mice were homogenized in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (1:20, w/v) using a Polytron homogenizer. Homogenates were sonicated (10 s, 3 \times), subjected to three freeze thaw cycles and centrifuged (8000 g, 4 $^{\circ}$ C, 15 min). The supernatant was added to a mixture of 0.167 mg/ml O-dianisidine hydrochloride and 0.075% H₂O₂. Increases in absorbance at 450 nm were monitored and activity was expressed as a rate of increase (U/mg protein). A minimum of three mice per group were measured.²⁷

Statistics

Data are presented as means \pm s.e. of at least three separate analyses. Data were analyzed by Student's *t*-test or ANOVA together with Newmann Keuls comparison were appropriate. $P < 0.05$ was set as the statistically significant difference level.

RESULTS

Increased Myeloperoxidase (MPO) in C-/P- Compared with C+/P+ Intestine

We first assessed the histological status of the C-/P- colons compared with control C+/P+ colons, under basal non-stimulated conditions. As illustrated in Figure 1b, the C-/P- colons showed substantial inflammatory infiltrate with no reduction in goblet cells or elongation of crypt depth in comparison with controls C+/P+ (Figure 1a). The specific activity of MPO, a marker of neutrophil infiltration was increased 18-fold in epithelia of colonic mucosa from the C-/P- mice compared with controls (16 \pm 3 vs 0.9 \pm 0.5, $n = 4$, $P < 0.05$). (Figure 1c). MPO activity was also increased in jejunal epithelial cells from C-/P- mice compared with the jejunum of control C+/P+ mice (Figure 1c). These findings suggested to us that both the small intestine and colon of the C-/P- mice had an increased amount of neutrophil infiltrate compared with age-matched control C+/P+ mice, under basal non-stimulated conditions.

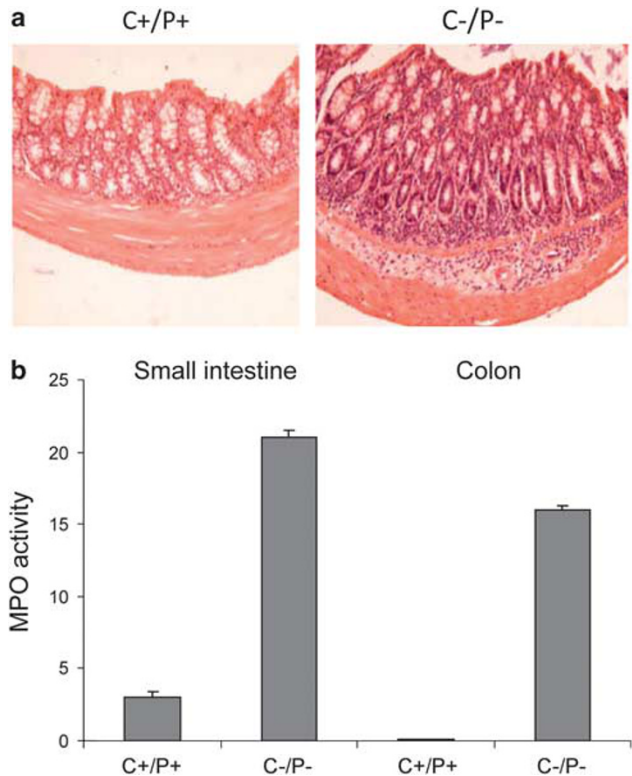


Figure 1 Increased inflammatory infiltrate and myeloperoxidase (MPO) activity in C-/P- mice compared with C+/P+ mice, under basal non-stimulated conditions. Light microscopic H&E sections of normal (C+/P+) colon at $\times 10$ (a). Light microscope H&E section of C-/P- colon at $\times 10$ (b) showing extensive inflammatory infiltrate. MPO activity, measured as in Egbuna *et al.*,²¹ in jejunum and colon of C+/P+ mice compared with C-/P- mice. * $P < 0.05$, $n = 3$.

Increased Wnt3a, CyclinD1, and MMP-7 in C-/P- Colons

Increased Wnt/ β -catenin activity in colonic crypts is usually inferred from increases in proliferative markers. We first prepared RNA from epithelial cells of C-/P- colons and C+/P+ colons and screened by RT-PCR several Wnt ligands and observed increases in the Wnt3a amplicons in the C-/P- colons. We confirmed by western blotting of isolated colonic epithelia that Wnt3a was increased seven-fold ($P < 0.05$, $n = 4$) in the C-/P- colons compared with age-matched C+/P+ controls (Figure 2a). As Wnt3a is well characterized to stimulate Wnt/ β -catenin signaling, we assessed whether the matrix metalloproteinase, MMP-7, was altered in the C-/P- colons. Matrilysin/MMP-7 was increased in the C-/P- colons eight-fold ($P < 0.05$, $n = 4$) in comparison with C+/P+ colons (Figure 2b). Another downstream target of Wnt/ β -catenin signaling is CyclinD1. CyclinD1 protein was increased three-fold ($P < 0.05$, $n = 4$) in the C-/P- colon compared with the C+/P+ controls (Figure 2c). Together these findings suggested to us that Wnt/ β -catenin signaling was increased in the colons of C-/P- mice compared with age-matched control colons.

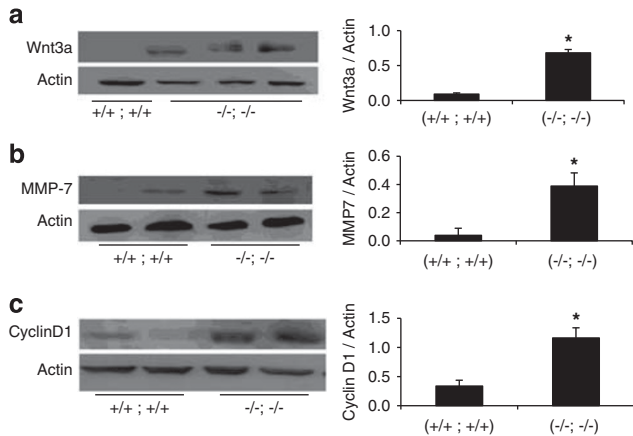


Figure 2 Western analyses of Wnt3a, Cyclin D1, and MMP-7 in C-/P- and C+/P+ colonic epithelia. (a) Wnt3a increased seven-fold in C-/P- compared with C+/P+. (b) MMP-7/Matrilysin increased eight-fold in C-/P- colonic epithelia compared with C+/P+ epithelia. (c) Cyclin D1 increased three-fold compared with C+/P+ epithelia. All increases significant ($P < 0.05$, $n = 5$).

Decreased Wnt5a, Ror2 and Increased TNFR1 in C-/P- Colons

Next, we assessed the CaSR-mediated determinants of non-canonical Wnt signaling in the C-/P- colons. As illustrated in Figure 3, Wnt5a was diminished four-fold ($P < 0.05$, $n = 4$) in the C-/P- colons in comparison with age-matched C+/P+ control colon scrapings (Figure 3a). In addition, the Wnt5a receptor, Ror2, was diminished 11-fold ($P < 0.05$, $n = 4$) in the C-/P- colons compared with control colons (Figure 3b). Previous *in vitro* analyses suggested Wnt5a/Ror2 activation would diminish TNFR1, therefore, we also assessed the status of TNFR1 in the double knockout colons. We found that TNFR1 was increased three-fold ($P < 0.05$, $n = 4$) in the C-/P- colonic scrapings compared with age-matched control colons (Figure 3c). These findings suggested that when the capacity for non-canonical Wnt signaling through Wnt5a/Ror2 engagement are reduced, targets such as TNFR1 undergo a compensatory increase.

Effect of AOM on C-/P- Colons

The colons of the C-/P- mice treated with AOM were not different in length than control C+/P+ mice. Interestingly, the number of ACF/cm² was higher in the C-/P- mice compared with control C+/P+ or C-/P- mice, which received saline injection (Figure 4). C-/P- mice that received AOM had 21.1 ± 3.8 ACF/cm². This increase was greater than in control C+/P+ mice injected with AOM (2.6 ± 0.8, $P < 0.0001$, $n = 6$). Similarly, the C-/P- colons, which were injected with saline, had the same number of ACF/cm² as control mice treated with AOM (5.4 ± 0.9 vs 2.6 ± 0.8, $n = 6$, NS). These findings indicate that ACF production was present in the unchallenged C-/P- colon and that AOM induces more ACF/cm² in the C-/P- colon compared with C+/P+ controls.

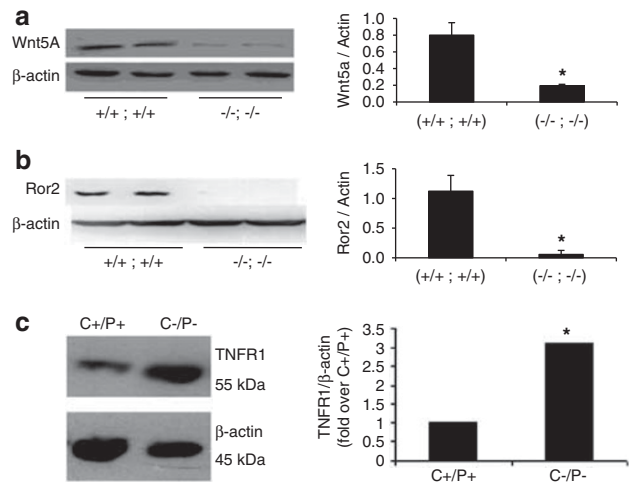


Figure 3 Western analyses of Wnt5a, Ror2, and TNFR1 in C-/P- and C+/P+ colonic epithelial cells. (a) Wnt5a reduced four-fold in C-/P- compared with C+/P+ colonic epithelia. (b) Ror2 expression reduced 11-fold in C-/P- compared with C+/P+ colonic epithelia. (c) TNFR1 increased three-fold in C-/P- compared with epithelia of C+/P+ mice. All changes significant. ($P < 0.05$, $n = 6$).

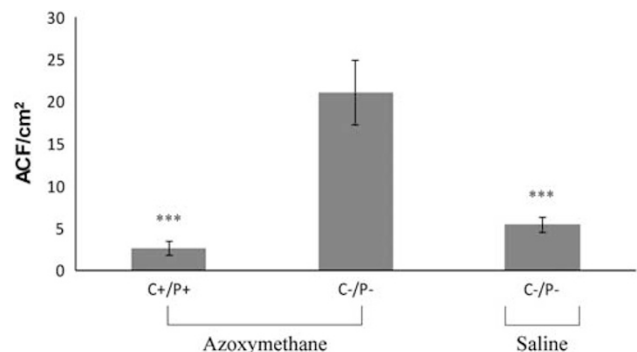


Figure 4 Aberrant crypt foci (ACF) increased in C-/P- colons. ACF/cm² in C+/P+ after azoxymethane (AOM; 15 mg/kg), C-/P- after AOM (15 mg/kg), C-/P- after saline injection. Total ACF counted per colon then expressed per colonic length.^{23,47,48} C-/P- with saline and C+/P+ with AOM were not different but both were significantly different compared with C-/P- with AOM injection. * $P < 0.0001$, $n = 6$.

DISCUSSION

The current report based on 'rescued' CaSR/PTH knockout mice demonstrates the colons of these mice have increased neutrophil infiltration, increased Wnt/ β -catenin signaling, ACF, and reduced non-canonical Wnt signaling. Following AOM injection, the C-/P- colons generated eight times more ACF than control C+/P+ colons. Together these data suggest the colonic CaSR can regulate levels of Wnt/ β -catenin signaling in colonic epithelia and protect against a carcinogen-induced crypt hyperproliferation. Furthermore, the substantial intestinal inflammation present in the double knockout colons suggests the CaSR may have a role in limiting inflammation throughout the intestine.

The stages in the development of colon cancer have been clearly elucidated.^{28–30} Increases in Wnt/ β -catenin signaling occur to drive hyperproliferation of crypts, which can manifest as ACF. AOM is a well-characterized agent used to generate ACF in models of colitis-associated colon cancer and to screen agents, which protect against ACF formation.^{25,26} In the current experiments, we detected the same number of ACF in saline-injected C – /P – colons as in the control C + /P + colons that received AOM. The number of ACF/cm² in the C + /P + mice were consistent with previous reports for a dose of 10 mg/kg of AOM.^{24–26} Although many of the determinants of crypt hyperproliferation are currently being defined, it is intriguing that the unchallenged C – P – colons had evidence of elevated Wnt/ β -catenin signaling. In the present study, the C – /P – colons had an increase in Wnt3a, a Wnt family member well characterized in stimulating the canonical β -catenin-dependent Wnt-signaling pathway.²⁸ The source of the increased Wnt3a in the C – /P – colonic epithelia is unknown. Previous *in situ* analysis of mouse colon did not report Wnt3a transcripts.³¹ Indeed pericryptal myofibroblasts when treated with CaSR activators showed reductions in Wnt3 and Wnt3a transcripts.¹³ A potential source of increased Wnt3a might be infiltrating macrophages¹⁵ and future experiments will address the source of Wnt3a. Both Wnt5a and Wnt3a may be secreted from macrophages,¹⁵ but the determinants of which Wnt ligand is secreted are not yet known.

Evidence that Wnt/ β -catenin signaling was increased in the C – /P – colonic epithelia was supported by the increases of two β -catenin-target genes: CyclinD1 and matrilysin (MMP-7). β -catenin has been shown to regulate CyclinD1 in colorectal cell lines *in vitro*.^{32,33} CyclinD1 overexpression has been observed in human colorectal cancer and in adenomas arising in the APC^{min/+} mouse.^{34,35} However, there is also evidence that CyclinD1 is not an immediate target of β -catenin following APC loss in the intestine,³⁶ suggesting increases in CyclinD1 were important for progression rather than initiation of colon cancer. Nevertheless, the C – P – colon showed a significant increase in CyclinD1 protein, consistent with increased cell proliferation. Matrilysin (MMP-7) is usually found in the Paneth cells of the small intestine,³⁷ but is overexpressed in about 80% of colorectal carcinomas. MMP-7 has a single TCF-4 site on its promoter and is expressed in some polyps before onset of the invasive phenotype. MMP-7 also is required for the invasive and metastatic potential of cancer cells.^{38–41} The increased MMP-7 expression found in the colonic epithelia of the C – /P – mice are therefore consistent with an increase in Wnt/ β -catenin signaling in the colon of these mice.

Although the C – /P – colons showed increased Wnt3a, CyclinD1, and MMP-7 protein expression in comparison with C + /P + colons, our phenotype did not reveal increased crypt depths. This is in contrast to the findings using the conditional intestinal knockout mouse,²⁰ where crypt depth and the total number of cells/crypt were increased.

Indeed, the study demonstrated enhanced β -catenin nuclear localization with expansion of the proliferative zone of the crypts. Our current findings based on the C – /P – colons therefore extended the studies using conditional knockout colons²⁰ by showing that two targets of Wnt/ β -catenin signaling, CyclinD1, and MMP-7 are increased. Furthermore, Wnt3a, a well-characterized activator of Wnt/ β -catenin signaling is also increased in the C – /P – colons.

The C – /P – colonic phenotype provided reduced amounts of Wnt5a and its receptor, Ror2. Previous *in vitro* studies have shown that on normal colonic myofibroblasts CaSR stimulation will increase Wnt5a secretion.¹³ When the CaSR is stimulated on the overlying colonic epithelia, Ror2 is increased.¹³ The engagement of Wnt5a with Ror2 has been shown to increase CDX2, sucrase-isomaltase, and villin.^{13,14} On the other hand, Wnt5a/Ror2 engagement will reduce TNFR1 expression to a level that prevents exogenous TNF α from initiating barrier damage to model membranes.¹⁵ In that context, we noted that with the reductions in Wnt5a and Ror2 in the C – /P – colons, TNFR1 was increased three-fold over its level in C + /P + colons. The increase in neutrophils in the C – /P – colons implies that neutrophil recruitment factors such as KC/CXCL1, MIP2/CXCL2, and LIX/CXCR5 have increased.⁴² These ligands are known to activate macrophages to secrete TNF α and increase neutrophil recruitment through activation of CXCR2.^{42,43} TNFR1 is the receptor responsible for mediating some of these effects, as TNFR1 – / – mice usually show reductions in KC.⁴² *Ex vivo* measurements of colonic TNF α in C + /P + and C – /P – mice demonstrated no difference between the two groups under basal, non-stimulated conditions, whereas TNFR1 was increased in the C – /P – colonic epithelia.⁴⁴ Indeed, models of pulmonary LPS tolerance have demonstrated that reductions in TNF α generate increased soluble TNF receptors, but changes in TNFR1 were not documented.⁴⁵ Further experiments are required to define whether the C – /P – phenotype is associated with alterations in these chemokines and CXCR2. The C – /P – mice did not exhibit loose stools or have detectable diarrhea compared with the C + /P + controls, unlike models of the development of colitis.⁴⁶ It will be of interest to examine whether colonic permeability is altered when the CaSR is absent from the colon. We do not know whether the increased TNFR1 in the C – /P – colons is due to alterations in CXCR2 or Wnt5a/Ror2 signaling, and further experiments will distinguish between these two types of signaling in the *in vivo* context. Consequently, we conclude that reductions in Wnt5a and Ror2 in the C – /P – colons are consistent with *in vitro* evidence that CaSR activity will regulate this non-canonical Wnt pathway.

Histopathological analyses did not show a difference in the number of goblet cells between the two groups of mice. The increased neutrophil presence in the small intestine and colon of the C – /P – may reflect a more proximate effector in the gut. Earlier studies had shown the presence of CaSR

transcripts in the Brunner's gland of the rat.^{47,48} As mucin is secreted by activation of the Brunner's gland in the duodenum, we speculate that the absence of the CaSR has altered Brunner's gland function/secretion in the C-/-P- animals. A reduction in mucin throughout the lumen of the GI tract could potentially allow more microbial influx across the intestinal epithelia. Increased bacterial transit may recruit more neutrophils to the intestine. We therefore hypothesize a reduction in mucin secretion from CaSR-stimulated Brunner's glands due to altered CaSR function can lead to increased inflammation. Further experiments are required to test this hypothesis.

What is the relationship between the increased colonic inflammation in the C-/-P- mice and the presence of ACF in the saline-injected animals? The colon is understood to be an organ in a constant state of inflammation.⁴⁹ Extensive evidence⁵⁰⁻⁵² supports the concept that inflammation promotes colon and other cancers. Indeed, recent studies have shown that chronic NF- κ B stimulation promotes the loss of APC.⁵³ Colitis-associated cancer requires dysplasia appearing and evolving to carcinoma,⁵⁴ with repeated episodes of severe inflammation generating mutations and methylation to result in high multifocal dysplasia and cancer. In contrast, sporadic colon cancer, a disease of defective Wnt/ β -catenin signaling, evolves from one or two foci.²⁸ The interplay of inflammation with Wnt/ β -catenin signaling is complex, and studies have demonstrated NF κ B enhancement of β -catenin signaling in villus cells allowed a dedifferentiation program with induction of crypt stem cells in an aberrant position.⁵⁵ Indeed, *in vitro* analyses have shown that IKK β will recruit CREB-binding protein with β -catenin as well as RelA/p65 with β -catenin to increase Wnt/ β -catenin reporter activity, establishing a direct mechanism to explain amplified Wnt/ β -catenin signaling. In the C-/-P- colonic epithelia, Wnt3a protein is increased. If this ligand is responsible for the increased Wnt/ β -catenin signaling (increases in MMP-7 and CyclinD1), then further stimulation of NF κ B by inflammatory mediators could amplify this signaling cascade to stimulate development of ACF. Such interplay was suggested by studies showing that MPO-positive cell number was increased in dysplasia in ACF and in adenomas although the highest found in colorectal cancer.⁵⁶ Animal models using the AOM/DSS model report variable results with ACF production and inflammation. For example, disruption of NT/NTR-1 signaling reduced the number of adenomas but not the number of ACF, suggesting NT promoted conversion of preneoplastic ACF into adenomas.⁵⁷ In contrast, chronic inflammation induced by Stat3 deletion in macrophages caused crypt hyperplasia, dysplasia (including irregularly branched crypts such as found in ACF), and an invasive carcinoma.⁵⁸ Nevertheless, current studies suggest inflammation may amplify activated Wnt/ β -catenin signaling.^{53,55} We therefore hypothesize that the increased inflammation in the C-/-P- colonic epithelia amplifies the increased Wnt/ β -catenin signaling we have

documented, and that the presence of ACF in the unchallenged, saline-injected C-/-P- colons are a consequence of these increases.

The current experiments have not tested a role for PTH in the AOM-induced ACF. Our heterozygotes, C+/-P-, did not breed, so we were unable to re-derive these mice to colony strength. Nevertheless, a role for PTH in colon cancer is controversial. Earlier studies demonstrated exogenous PTH had no effect on ACFs induced in the colons of AOM-injected rats.⁵⁹ Latter *in vitro* studies demonstrated exogenous PTH caused G₀/G₁ cell cycle arrest in an adenocarcinoma cell line.⁶⁰ Primary parathyroidism has been associated with colon cancer,⁶¹ and one explanation for the effect proposes reduced colonic luminal Ca²⁺ because of the stimulatory effect of PTH on 1,25-dihydroxyVitaminD₃. The stimulation of 1,25-dihydroxyVitaminD₃ was thought to increase duodenal Ca²⁺ absorption to a degree that lowered colonic lumen Ca²⁺ levels, which presumably would reduce CaSR activation.⁶² Although interesting, this hypothesis may be difficult to reconcile with the VDR elements in the CaSR promoter that when activated may increase CaSR expression.⁶³ Indeed, more recent studies have shown that high serum levels (>65 ng/l) of PTH are associated with increased colorectal cancer risk in men.⁶⁴ As the C-/-P- mice have no PTH, it is difficult to reconcile these results with our findings, which would suggest lower circulating PTH levels would protect against ACF development. Clearly additional work with PTH heterozygotes and knockout mice are needed to address this issue.

In summary, AOM stimulated eight-fold more ACF/cm² in the double knockout C-/-P- colons compared with age-matched C+/-P+ colons. Indeed, saline-injected C-/-P- colons had the same number of ACF/cm² as age-mated C+/-P+ colons injected with AOM. The presence of increased Wnt/ β -catenin signaling in the C-/-P- colons was demonstrated by increased Wnt3a, CyclinD1, and MMP-7 protein compared with the C+/-P+ colonic epithelia. Non-canonical Wnt signaling manifested by Wnt5a/Ror2 protein levels were substantially reduced in the C-/-P- mice and TNFR1 was increased in a compensatory fashion. The C-/-P- intestines also had an extensive neutrophil infiltration in both the small intestine and colon. The global 'rescued' C-/-P- knockout colon had increased inflammation and increased Wnt/ β -catenin signaling. Presumably, both of these effects contributed to the generation of more ACF/cm² in response to the AOM injections. The increased inflammation, Wnt/ β -catenin signaling and ACFs in the C-/-P- colons suggest this model can be used to determine whether Western-style diets alter the development of intestinal adenomas in the absence of colonic CaSR or how the CaSR effects development of colitis-associated cancer. We conclude that the colonic CaSR regulates Wnt/ β -catenin signaling in the intact colon and protects against the formation of preneoplastic ACF. Therefore, it is highly likely that when increased dietary calcium and dairy products chemoprotect

against the development of colorectal cancer, it is the colonic CaSR that is being stimulated.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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