# Parallel Elevation of Colonic 1,25-Dihydroxyvitamin $D_3$ Levels and Apoptosis in Female Mice on a Calcium-deficient Diet

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**Abstract.** *Background: Epidemiology suggests that nutritional calcium and vitamin D together prevent colorectal tumor progression. 1,25(OH)$_2$D$_3$ is synthesized and degraded in colonocytes and, when bound to its receptor, has antiproliferative activity. Materials and Methods: 1,25(OH)$_2$D$_3$ levels have been successfully measured in cell culture, but this is technically difficult in tissues. Double extraction coupled to an enzyme immunoassay was used to determine 1,25(OH)$_2$D$_3$ concentration in colon mucosa. Results: In a mouse model fed low (0.04%) nutritional calcium, expression of the vitamin D catabolizing CYP24A1, of the synthesizing CYP27B1 hydroxylase and of the vitamin D receptor was induced in the right colon only. While CYP24A1 mRNA was raised in both genders, raised CYP27B1 and VDR was found in females only. Levels of 1,25(OH)$_2$D$_3$ were significantly higher in the right colon of females fed 0.04% calcium compared with the control group on 0.9% calcium, and with males fed either diet. Parallel to increased 1,25(OH)$_2$D$_3$, the intrinsic apoptotic pathway was enhanced in the right colon of females only. Conclusion: This demonstrates the significance of high nutritional calcium for colonic accumulation of 1,25(OH)$_2$D$_3$ and suggests that female sex hormones may protect against mitotic action of low nutritional calcium by inducing 1,25(OH)$_2$D$_3$ synthesis.*

Epidemiological data have demonstrated that colon cancer incidence is inversely correlated with indices of vitamin D status, such as ultraviolet exposure (1). At least 80% of requisite vitamin D is formed within the skin by UV-B energy from the precursor 7-dehydrocholesterol while nutritional contribution is low. The serum level of the 25-hydroxylated vitamin D precursor 25(OH)$D_3$ is a measure of vitamin D deficiency (commonly accepted to be below 30 nmol/l) and sufficiency (above 70 nmol/l) (2). A serious lack in vitamin D can be found in the general population during winter, and all year round in the elderly. Dark-skinned people produce less vitamin $D_3$ since melanin, the main pigment of the skin, functions as a very efficient filter against UV-B radiation. There is a 'grey' zone of vitamin D insufficiency (between 30 and 70 nmol/l) that might be related to increased occurrence of sporadic malignancies (3).

The vitamin D receptor (VDR) is ubiquitously expressed in the human body (for a review see *e.g.* (4)). Although its relevance is not entirely clear yet, its importance for normal human physiology must necessarily be high. There is evidence from the three organs most affected by sporadic, advanced age-related cancer: the mammary gland (5), the prostate gland (6) and the colon (7), that a functional VDR bound to its natural ligand 1,25-dihydroxyvitamin $D_3$ (1,25(OH)$_2$D$_3$) exerts negative growth regulatory effects and contributes to maintenance of the normal differentiated phenotype. However, it has to be recognized that serum concentrations of 1,25(OH)$_2$D$_3$ are at picomolar levels, but nanomolar levels are necessary to influence proliferation, differentiation and apoptosis of cells (8).

Also the synthesizing 25-vitamin$D_3$-1α-hydroxylase (CYP27B1) is expressed in many tissues, among them the colonic mucosa and, with hyperproliferation during premalignancy, its expression is enhanced in parallel to that of the VDR (9). Since it became evident from *in vitro* work that this CYP27B1 activity in extrarenal cells could result in synthesis of considerable amounts of 1,25(OH)$_2$D$_3$, it was suggested that accumulation of the active vitamin D metabolite in various tissues could reach levels that would locally result in antimitotic, proapoptotic activity (10). This however remained a theory due to technical problems in evaluation of 1,25(OH)$_2$D$_3$ in tissue samples.

In this paper, we not only provide evidence in a mouse model that 1,25(OH)$_2$D$_3$ can indeed be measured in colonic mucosa, but also that synthesis is modulated by nutrition.

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*Key Words:* Colonic 1,25(OH)$_2$D$_3$ concentration, gender-dependent, dietary calcium, apoptosis, vitamin D hydroxylases.

## Materials and Methods

C57BL/6 mice were housed at the Centre for Laboratory Animal Care at the University of Vienna in a closed environment. Mice were weaned when three weeks of age, and were then fed *ad libitum* a standard diet (basis was AIN 76A) containing either high (0.9%) or low levels (0.04%) of calcium (Harlan Teklad Europe, Borchen, Germany). The calcium levels chosen represent the equivalent of high and low calcium intake in humans (11). Treatment groups consisted of at least 5 animals. All animals were sacrificed when 13 to 14 weeks old. Mice were killed by cervical dislocation and tissue samples were collected. The colon (without coecum) was divided into right (from end of coecum to middle of transversum) and left (from middle of transversum to rectum) parts, the lumen was cut open, rinsed with phosphate-buffered saline (PBS), the mucosa was scraped off on ice and was frozen immediately in liquid nitrogen. An aliquot of fresh mucosa from each animal and colon segment was used for apoptosis measurements. Study protocols were reviewed and approved by the Institutional Committee of Animal Experimentation of the Medical University of Vienna and by the Austrian Ministry of Science and Education.

*Reverse transcriptase–polymerase chain reaction (RT-PCR).* Total RNA was prepared with TRIzol™ reagent (Invitrogen, Lofer, Austria) according to the manufacturer's instructions. A total of 2 μg of RNA were reverse-transcribed (RevertAid™ H Minus M-MuLV Reverse Transcriptase; Fermentas, St. Leon-Rot, GER) using random hexamer primer at 42˚C for 60 min, 45˚C for 10 min, followed by 72˚C for 15 min.

*Quantitative real time RT-PCR. VDR, CYP27B1* and *CYP24A1* mRNA levels were quantified by the comparative ΔΔCT method. For each experimental sample, the relative abundance value obtained was normalized to the value derived from the endogenous control (18S rRNA) of the corresponding sample. cDNA from a pool of C57BL/6 mouse colonic mucosa (a mixture of right and left parts) was designated as the 'calibrator' and the relative expression levels of all other samples were assessed relative to the calibrator. The real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Triplicates were set up for each sample and transcript under investigation. PCR conditions were: 50˚C for 2 min, 94˚C for 2 min, which was followed by 40 cycles of 94˚C for 15 s and 60˚C for 30 s. Primers and internal probes were designed using Primer Express (Applied Biosystems) and were located on different exons to prevent amplification of contaminating genomic DNA. For detection of 18S rRNA, the VIC-labelled Assays-on-DemandTM Gene Expression kit (Applied Biosystems) was used. The sequences of *VDR, CYP27B1*, and *CYP24A1* were described elsewhere (12).

*Apoptosis measurement.* Apoptosis was measured with the APO-ONE® Homogeneous Caspase-3/7 Assay (Promega Corporation, Madison, WI, USA), following the manufacturer's instruction. This assay is based on the ability of caspase-3/7 (from the tissue homogenate) to cleave the non-fluorescent caspase substrate Z-DEVD-R110, creating the fluorescent rhodamine 110 which is detected at 499/521 nm. The Homogeneous Caspase-3/7 Reagent was prepared by diluting the substrate 1:100 with the assay buffer and was added to the homogenized mouse colonic mucosa in a 1:1 ratio. Caspase activity was measured after 3 hours' incubation and was related to the amount of protein found in the mucosal homogenate. Protein concentration was measured with the BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA).

*Evaluation of 1,25(OH)$_2$D$_3$ concentration in colonic mucosa.* Frozen mucosa was reconstituted in PBS, homogenized for 1 min on ice with an Ultra Turrax (Janke&Kunkel IKA®-Labortechnik, Staufen, Germany) and was transferred into glass tubes. An aliquot of each homogenate was used to measure protein concentration by the BCA™ Protein Assay Kit (Thermo Fisher Scientific). Extraction of the lipophilic fraction is described elsewhere (13). Briefly, 2 ml dichloromethane ($CH_2Cl_2$) were added to 2 ml extract followed by centrifugation at 1,500 rpm for 10 min. The lower phase was collected and re-extracted twice with $CH_2Cl_2$. Extracts were dried under a gentle stream of nitrogen at 55˚C and were reconstituted in propylene glycol. PBS containing 2% bovine serum albumin (BSA) was added and samples were processed for immunoextraction and 1,25(OH)$_2$D$_3$ measurement.

1,25(OH)$_2$D$_3$ levels were measured with a 1,25dihydroxyvitamin D enzyme immunoassay (EIA) from Immunodiagnostic Systems Inc., Frankfurt am Main, Germany (IDS). This assay uses solid-phase immunoextraction and colorimetric detection. Although DEQAS was established to compare worldwide differential methods for 25(OH)D evaluation (14) and reasonable consensus has been achieved, assay performance for 1,25(OH)$_2$D$_3$ is still far from being convincing. Recently, the well-established thymus radioreceptor assay for 1,25(OH)$_2$D$_3$ was compared with the EIA from IDS. Between-run differences were 15-20% and other methods are unlikely to provide better performance (15). Therefore, after tissue (or cell pellet) extraction, the EIA from IDS was used following the manufacturer's instructions. The colour intensity of the assay was inversely proportional to the concentration of 1,25(OH)$_2$D$_3$. Tissue 1,25(OH)$_2$D$_3$ concentrations were calculated with respect to the protein amount of the extract and were expressed as fmol/mg. Assessment of protein concentration was based on measurement of homogenate aliquots with the BCA™ Protein Assay Kit.

*Validation of the method.* Caco-2 cells were harvested and homogenized as described elsewhere (13). These cells were cultured serum-free and were not exposed to the vitamin D precursor, therefore they did not express any endogenous 1,25(OH)$_2$D$_3$. Protein was determined with the BCA™ kit and adjusted to 1.5 mg/ml with PBS, then 4 μl of a 1 nM 1,25(OH)$_2$D$_3$ stock solution were added per 1 ml sample for it to be in the optimal range of assay sensitivity (exact concentration of added 1,25(OH)$_2$D$_3$ was determined by UV absorbance at 265 nm). The mean percentage recovery of exogenous 1,25(OH)$_2$D$_3$ was 3.4 pmol/l (±0.37, n=5), *i.e.* 85%. Such recoveries are similar to those obtained and published recently with the EIA from IDS (15).

## Results

*Site- and gender-specific expression of CYP27B1 and VDR mRNA is regulated by dietary calcium in the mouse colon. VDR* and *CYP27B1* mRNA were evaluated by real time RT-PCR in mice fed either a low or high calcium diet. In the right colon of the controls, *VDR* expression was higher in females than in males. When mice were fed a low calcium diet, *VDR* mRNA expression became even higher in females, although narrowly missing significance. In the left colon of the control mice, the differences in *VDR* levels between females and males were even more pronounced ($p \leq 0.05$). However, in contrast to that in the right colon, this elevated expression was abolished when mice were fed the low calcium diet (Figure 1A).

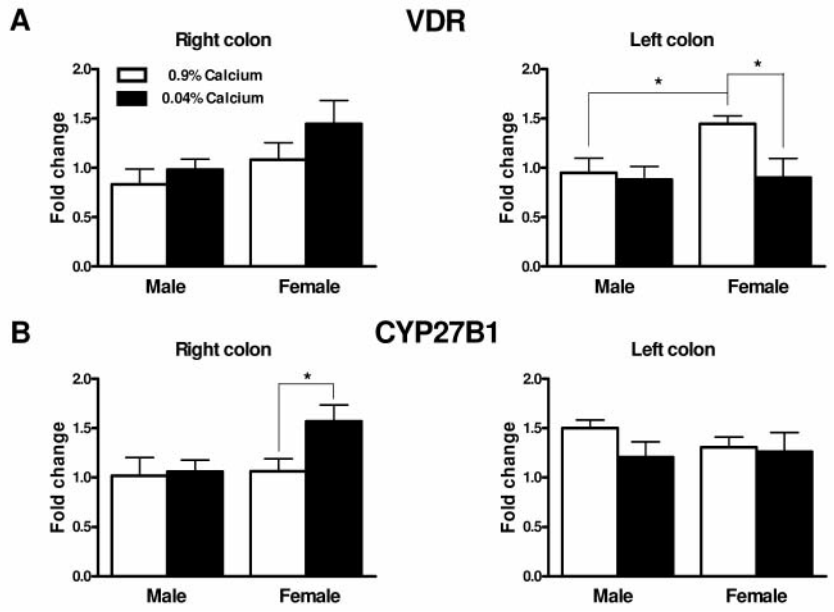


Figure 1. *The mRNA expression levels of VDR (A) and CYP27B1 (B) in the colonic mucosa of C57BL/6 mice fed either with 0.04% or 0.9% calcium in their diet. Data were derived from real-time RT-PCR and were analyzed by the comparative ΔΔCT method. Results are expressed as mean fold change ± S.E.M. (n=5 mice/group) and are presented according to site (right (RC) and left (LC) colon) and gender. For statistical analysis, Student's t-test (two-tailed) was applied. Values of p≤0.05 were considered statistically significant and are marked with an asterisk.*

We also observed site-specific differences in *CYP27B1* levels: Expression of *CYP27B1* mRNA coding for the synthesizing vitamin D hydroxylase was higher in the left than in the right colon of mice on the control diet ($p \leq 0.01$, n=9). However, we did not observe any gender-specific differences of *CYP27B1* levels in the control groups. In contrast, low dietary calcium elevated expression of *CYP27B1* mRNA was found solely in the right colon of female mice (Figure 1B).

*Site- and gender-dependent expression of CYP24A1 mRNA.* *CYP24A1* mRNA was practically undetectable even by real-time RT PCR in colonic mucosa of mice on a normal 0.9% calcium diet. When animals were fed a diet containing only 0.04% calcium, *CYP24A1* mRNA expression was increased, especially in male mice, however, only in the right colon (Figure 2), whereas expression in the left colon remained exceedingly low (not shown).

*Site- and gender-specific synthesis of $1,25(OH)_2D_3$ in colonic mucosa is influenced by dietary calcium.* $1,25(OH)_2D_3$ accumulation in mouse colonic mucosa was measured. Interestingly, these concentrations were very similar, regardless of gender and site, and were an average of 1.5 fmol/mg protein (SD: ±0.6 fmol/mg) when mice were on a high calcium diet. However, when we compared this with the levels measured in the colonic mucosa of mice on a low calcium diet, we found a significant elevation of $1,25(OH)_2D_3$ in the mucosa derived from the right colon of female mice only (Figure 3A, up to 2.7 fmol/mg), while $1,25(OH)_2D_3$ levels remained low in the left colon regardless of the calcium content of the diet (not shown).

*Site- and gender-specific regulation of apoptosis by dietary calcium in mouse colon.* Apoptosis was measured with the APO-ONE® Homogeneous Caspase-3/7 Assay in mucosal homogenates from the same mice that were used for $1,25(OH)_2D_3$ measurement. When we compared apoptosis in the right and left colon-derived mucosa, no significant difference was apparent between males and females on the control diet (Figure 3B). However, low dietary calcium increased caspase activity significantly, but only in the right colon of female mice (Figure 3B).

## Discussion

In this paper, the actual quantification of the active vitamin D metabolite $1,25(OH)_2D_3$ in colonic mucosa is described for the first time. While DEQAS has extensively monitored performance of assays for 25(OH)D in serum, evaluation of $1,25(OH)_2D_3$ is more difficult, most likely due to the at least 1,000-fold lower levels of the active metabolite there. By coupling two different extraction methods, we were able to demonstrate that quantification of the concentration of $1,25(OH)_2D_3$ in tissues or cells is possible with an average yield of 85%. While, in the past, our laboratory as well as several others were able to evaluate either expression of the synthesizing (CYP27B1) and of the catabolizing (CYP24A1)

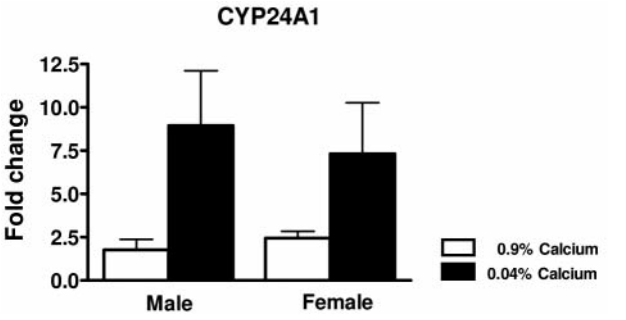


Figure 2. *Comparison of CYP24A1 mRNA expression in the colonic mucosa of C57BL/6 mice fed either with 0.04% or 0.9% calcium. Data were obtained by real-time RT-PCR and were quantified by the comparative ΔΔCT method. Results are shown as mean fold change ± S.E.M. for the right colon only of female and male animals (n=5 mice/group). Expression in the left colon was exceedingly low (not shown). Statistical significance was evaluated by two-tailed Student's t-test and was not quite reached due to data variation.*

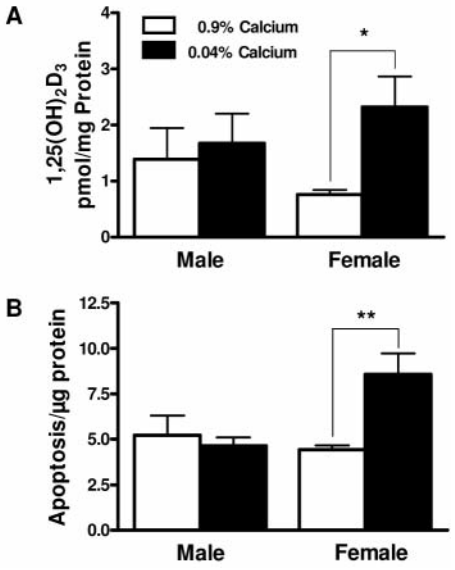


Figure 3. *Quantification of 1,25(OH)$_2$D$_3$ levels (A) and apoptosis (B) in the colonic mucosa of C57BL/6 mice fed either with 0.04% or 0.9% calcium in their AIN76 diet. An ELISA-based assay was used to evaluate 1,25(OH)$_2$D$_3$ levels (see Materials and Methods section). Apoptosis was assessed by measuring caspase-3/7 activity. Results were normalized with respect to total protein amount of the respective colonic mucosal sample and are shown with ± S.E.M. (n=5 mice/group) for the right colon of both genders only. Statistical significance of data was calculated with two-tailed Student's t-test: *p-value ≤0.05 and **p≤0.01.*

vitamin D hydroxylases (16), or the potential of cells to metabolise 25(OH)D$_3$ by HPLC (13), it was not possible to quantify 1,25(OH)$_2$D$_3$ produced in tissues. This is a point of primary importance in view of the assumption that it is the locally produced endogenous 1,25(OH)$_2$D$_3$ that is able to prevent colonic tumor progression. This assumption was based on our previous observation that expression of CYP27B1 protein in normal human colonic mucosa was scarce while during tumor pathogenesis, especially in early well-differentiated tumors, expression was increased sharply in many colon cancer patients, but then was almost eliminated in highly malignant tumors.

Since the discovery of Garland *et al.* (17) almost 30 years ago, that both calcium and vitamin D could modify incidence of colorectal cancer, this subject has been of primary interest for tumor prevention. However, several prospective studies provided no evidence for an inverse association between calcium or vitamin D intake and tumor incidence (see *e.g.* (18, 19)) or risk prevention was small (see *e.g.* (20, 21)). It was only when calcium plus vitamin D were given as a chemopreventive regimen to adenomatous polyp patients that several molecular changes contributing to reduced polyp formation were seen (22). The potentially most important phenomenon was an increase in expression of apoptotic markers. This provided experimental support for the suggestion that vitamin D and calcium were interacting as chemopreventive agents and led to further human trials supporting the claim that supplementation of both calcium and vitamin D could prevent cancer incidence much more effectively than either substance alone (see *e.g.* (23, 24)).

Using the mouse as a model to investigate a potential mechanism, we were able to demonstrate previously that a diet low in calcium (0.04 compared with 0.9%) enhanced proliferation of colonic mucosal cells, elevated expression of cyclin D1, reduced expression of the cyclin-dependent kinase inhibitor p21 and increased CYP24A1 expression (11). Numerous studies have provided evidence that women may be better protected against colorectal cancer pathogenesis than men. This implies that sex hormones might play a significant role in this process (see *e.g.* (25)). High bone mass density is a marker for cumulative exposure to endogenous estrogen, calcium and vitamin D intake and it is known that women with low bone mass density are more likely to develop a more aggressive form of colon cancer (26). It has been demonstrated by Schwartz *et al.* (27) that 17β-estradiol enhanced vitamin D receptor expression in a rat model. Estrogens may impact on breast cancer cell sensitivity to vitamin D through regulation of the *VDR* promoter (28). Our mouse model confirmed that the females express more *VDR* (Figure 1A). We showed recently in human colon cancer cells *in vitro* that supraphysiological concentrations of 17β-estradiol not only elevated *CYP27B1* mRNA and activity, but also reduced that of *CYP24A1* (29). This could conceivably result in enhanced accumulation of 1,25(OH)$_2$D$_3$ and could provide a means for curbing tumor progression.

However, when we evaluated and compared *CYP27B1* mRNA by real-time PCR in male and female mice, no significant difference was apparent. This may be due to the

low normal 17β-estradiol concentration in female mice, but also to the fact that *CYP27B1* in normal colon cells is exceedingly low and potential gender-related differences are hard to evaluate (16). Interestingly though, only in the right colon of female mice did low dietary calcium lead to a significant increase of expression of *CYP27B1*, and of *VDR* as well. A recent study by Liu and his colleagues (30) also suggests a protective role for *CYP27B1* (in this case against dextran sulphate sodium (DSS)-induced colitis). This study has shown that DSS-induced colitis stimulated expression of CYP27B1 in the proximal colon of wild-type C57BL/6 mice, while Cyp27b1$^{-/-}$ animals were more susceptible to DSS-induced colitis than wild-type.

In addition, *CYP24A1* mRNA expression in colonic mucosa was increased by low dietary calcium in the right colon only (Figure 2). However, CYP24A1 accumulation was independent of gender. Therefore, our data suggest a role for female sex hormones in the regulation of colonic CYP27B1 and VDR expression (whatever the relation to low dietary calcium might be), but none concerning CYP24A1 expression. In this respect, it might be of significance that bile acids such as lithocholic acid are implicated in the progression of colon cancer (31). Normally, dietary calcium may bind bile acids especially in the upper colon and eliminate them in the feces. Unbound lithocholic acid may bind to the VDR with low affinity and may induce, among others, enzymes of the CYP3A family known to catabolize xenobiotic substances. However, it also can induce *CYP24A1* (32), and this would be independent of gender.

Measurement of $1,25(OH)_2D_3$ in colonic mucosal homogenates clearly showed that low dietary calcium increased local concentration of the active vitamin D metabolite significantly, in the right colon of female mice only. In order to understand how low calcium activates vitamin D synthesis particularly in the right colon of the females, further investigation is necessary. There is controversy as to which estrogen receptor (ER) is primarily expressed in the colon (33, 34), and the actual colonic site-dependent distribution of the ER is yet unknown. However, there is some evidence, that ER-β-mediated processes may have a role in the prevention of colorectal cancer, supporting the hypothesis that there may be sex-specific differences in colorectal cancer pathogenesis.

Increased caspase activity could provide protection against potential hyperproliferation and we suggest that it occurs due to elevated tissue-localized concentrations of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ has been shown to activate the downstream effector protease caspase-3, and the upstream initiator protease caspase-9, the apical protease in the mitochondrial intrinsic pathway for apoptosis in, for instance, prostate cancer cells (35). In a mouse model for intestinal tumor pathogenesis, the effect of dietary $1,25(OH)_2D_3$ and calcium on apoptosis was recently demonstrated (36). While in these studies vitamin D comes from exogenous sources, apoptosis and action of tissue-localized $1,25(OH)_2D_3$ has not been studied before, except in rats with hyperplastic parathyroids. When vitamin D was directly injected into glands of live animals, apoptotic markers were increased after 24 h (37).

In summary, our results show that measurement of $1,25(OH)_2D_3$ in tissues is indeed possible with reasonable accuracy. *CYP27B1* and *VDR* mRNA expression are elevated specifically in the right colon of female mice fed a diet containing low calcium, and this dietary deficiency results not only in hyperproliferation of colonic crypts (12), but also leads to increased mucosal $1,25(OH)_2D_3$ accumulation and, in parallel, enhanced apoptosis. Importantly, we demonstrate that positive regulation of tissue-localized synthesis of the active vitamin D metabolite in colonocytes might contribute to regulation of signal transduction pathways relevant for prevention of colonic tumorigenesis.

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