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Genetic deletion of mPGES-1 accelerates intestinal tumorigenesis in APC$^{Min/+}$ mice

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ABSTRACT

The induced synthesis of bioactive prostanoids downstream of cyclooxygenase-2 (COX-2) and prostaglandin H_2 (PGH_2) exerts a critical event in colorectal carcinogenesis. Here we demonstrate that APC$^{Min/+}$ mice with genetic deletion of microsomal prostaglandin E synthase-1 (mPGES-1), which catalyses the terminal conversion of PGH_2 into PGE_2, surprisingly develop more and generally larger intestinal tumors than do mPGES-1 wild type littermates (mean number of tumors/intestine 80 vs. 38, $p < 0.0005$, mean tumor diameter 1.64 vs. 1.12 mm, $p < 0.0005$). No deviation regarding the expression of other PGE_2 related enzymes (COX-1, COX-2, mPGES-2, cPGES, and 15-PGDH) or receptors (EP1–4) was obvious among the mPGES-1 deficient mice. PGE_2 levels were suppressed in tumors of mPGES-1 deficient animals, but the concentrations of other PGH_2 derived prostanoids were generally enhanced, being most prominent for TxA_2 and PGD_2. Thus, we hypothesise that a redirected synthesis towards other lipid mediators might (over)compensate for loss of mPGES-1/PGE_2 during intestinal tumorigenesis. Nevertheless, our results question the suitability for mPGES-1 targeting therapy in the treatment or prevention of colorectal cancer.

Colorectal cancer is one of the leading malignancies in Western world, affecting about 1 000 000 new patients each year. It has for long been known that regular usage of non-steroidal anti-inflammatory drugs (NSAIDs) dramatically reduces the risk of developing colorectal tumors. This effect has mainly been attributed to the inhibition of cyclooxygenase-2 (COX-2), that is upregulated in inflammatory and neoplastic states and catalyses the synthesis of prostaglandin H_2 (PGH_2). Among other signalling molecules derived from PGH_2, prostaglandin E_2 (PGE_2) is present at high levels in colorectal tumors, and is thought to be essentially involved in intestinal carcinogenesis (reviewed in [1]) although recent reports suggest other bioactive PGH_2 metabolites to exert protumorigenic effects as well [2–10].

Though, treatment with NSAIDs or COX-2 selective inhibitors is associated with severe systemic side effects, most probably due to the altered balance of these non-PGE_2 metabolites downstream of COX generated PGH_2 [1]. In this context, the terminal prostaglandin E synthases provide attractive targets for specific modulation of PGE_2 production. In particular, microsomal prostaglandin E synthase-1 (mPGES-1) has attracted interest, since this isoform is markedly upregulated in neoplastic tissue, and thought to be critical for tumor PGE_2 generation [11,12], although this has not yet been fully assessed *in vivo*.

In the present study, the effect of homozygous mPGES-1 gene (PTGES) deletion was investigated with regard to intestinal tumor formation in APC$^{Min/+}$ mice. Notably, we found that the number and size of intestinal tumors were markedly increased in APC$^{Min/+}$ mPGES-1$^{-/-}$ mice, although PGE_2 levels were suppressed as expected. Enhanced concentrations of other COX-2 derived eicosanoids were present in tumors from mPGES-1 deleted animals, potentially (over)compensating for the loss of PGE_2. Nevertheless, our findings clearly challenge the suitability for mPGES-1 targeting therapy in the treatment or prevention of colorectal cancer.

Materials and methods

Animals. C57BL/6 mice with the APC$^{Min/+}$ genotype (The Jackson Laboratory, Bar Harbor, ME, USA), or C57BL/6 mice with targeted deletion of the mPGES-1 encoding gene (mPGES-1$^{-/-}$), previously described in [13], were used in the present study. APC$^{Min/+}$ and mPGES-1$^{-/-}$ mice were crossed, generating APC$^{Min/+}$mPGES-1$^{+/-}$ and APC$^{+/+}$mPGES-1$^{+/-}$ animals (F1 generation), which were subsequently intercrossed (F2 generation), yielding APC$^{Min/+}$mPGES-1$^{+/+}$, APC$^{Min/+}$mPGES-1$^{+/-}$ and APC$^{Min/+}$mPGES-1$^{-/-}$ littermates, which were included in the present analyses. Animals were housed in ventilated cages at 23 ± 1 °C with a 12-h light/dark cycle. Standard diet (CRME rodent, Special Diet Services Ltd., Witham, Essex, UK) and water were available *ad libitum*. DNA for genotyping was

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isolated from tail biopsies with the Extract-N-Amp Tissue PCR Kit™ (Sigma, St. Louis, MO, USA), according to supplier's recommendations. The APC and mPGES-1 encoding genes were genotyped as previously described [13,14]. All experimental procedures were approved by the animal Care and Use Committee at the Linköping University.

Tumor quantitation. At age 125 ± 5 days, mice were sacrificed with CO_2 and whole intestines were collected. Intestines were held on ice and immediately cut longitudinally and carefully rinsed with ice-cold 0.9% saline. Small intestines were subdivided in proximal, middle, and distal third, respectively. Tumor frequency and size were determined under a 20× dissection microscope (Leica Microsystems GmbH, Wetzlar, Germany) by a single examiner (NE) who was blinded to the genotype. Tumor size was approximated by measuring the maximum tumor diameter with a calibrated eyepiece reticule (Leica).

Histopathological classification. A subset of tumors were put in 4% formaldehyde solution and embedded in paraffin. Standard H&E staining was performed, and tumors were classified by an experienced pathologist (HO) who was blinded to the genotype.

RNA isolation. Tumors and non-tumor intestinal mucosa specimens were collected and immediately placed in RNAlater (Ambion Inc., Austin, TX, USA). Specimens were homogenised through shaking with 5 mm stainless steal beads (Qiagen, Hilden, Germany) in a TissueLyser (Qiagen) at 20 Hz for 2 × 2 min. RNA was then isolated with RNeasy Mini Kit (Qiagen) according to the supplier's recommendations. Total RNA concentrations were determined spectrophotometrically.

cDNA synthesis and real-time PCR. Five hundred nanograms of total RNA from each sample was reversely transcribed into cDNA with Superscript III (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. mRNA expression of mPGES-1, mPGES-2, cPGES, COX-1, COX-2, 15-PGDH, and PGE_2 receptors (EP) 1–4 was subsequently determined with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using predesigned primer/probe assays purchased from Applied Biosystems (sequences available at request). C_t values were related to the endogenous control gene GAPDH (ΔC_t), and relative expression ($2^{-\Delta Ct}$) was normalised to the average expression in non-tumor intestinal mucosa of $APC^{Min/+}$mPGES-$1^{+/+}$ mice ($2^{-\Delta\Delta Ct}$). To validate GAPDH as a reliable endogenous control gene, a randomly chosen subset of tumors and non-tumor mucosal specimens were co-analysed with regard to three control genes, namely GAPDH, β-actin, and β-glucuronidase (Applied Biosysems), revealing similar relationships between Ct values of the different genes among all specimens (data not shown).

Measurement of PGE_2, TxB_2, PGD_2-MOX, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$. Whole tumor specimens and adjacent mucosa samples were homogenised in 0.1 M phosphate buffer (pH 7.4, containing 1 mM EDTA and 10 μL indomethacin) through shaking in a TissueLyser (Qiagen, for details see above). Fractions of the lysate were diluted in EIA buffer and PGE_2, thromboxane B_2 (TxB_2), 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) concentrations were determined with appropriate Monoclonal EIA Kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's recommendations. With regard to PGD_2, conversion to a more stable methoxylamine (MOX)-derivative (PGD_2-MOX) was performed according to supplier's recommendations. All samples were analysed in duplicate and at least at two different dilutions to ensure reliable results within the spectrum of the assays (B/B_0 between 20% and 80%).

Statistics. Data were expressed as means ± standard error of the mean (SEM). For comparative analyses, Student's *t*-test was used. All calculations were performed with the SPSS Software 15.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Animal characteristics

At age 125 ± 5 days, mice were sacrificed and intestines were collected. With few exceptions, mice of all investigated genotypes survived until this time point, although the body weight was significantly lower among both male and female $APC^{Min/+}$mPGES-$1^{-/-}$ animals (Table 1).

Tumor multiplicity, size, and histopathology

$APC^{Min/+}$mPGES-$1^{+/+}$ mice displayed a total of 38.19 ± 2.22 (mean ± SEM, n = 20) intestinal tumors per mouse. Surprisingly, the intestinal tumor frequency was twofold elevated among $APC^{Min/+}$mPGES-$1^{-/-}$ animals, reaching 79.56 ± 9.21 ($n = 12$, $p < 0.0005$).

The increased tumor frequency among mPGES-1 deleted animals was observed in all parts of small intestine (proximal, middle, and distal third), with most of the tumors located in the distal third (Fig. 1A), and results were consistent among both males and females (data not shown). Colonic tumor frequency barely differed between genotypes (2.56 ± 0.72 mm [mean ± SEM] vs. 2.42 mm ± 0.33, $p = 0.836$, Fig. 1A).

Further, tumors were found to be significantly larger in small intestines of $APC^{Min/+}$mPGES-$1^{-/-}$ mice (1.64 ± 0.043 mm [mean tumor diameter ± SEM], n = 276) in comparison with $APC^{min/+}$mPGES-$1^{+/+}$ littermates (1.12 ± 0.034 mm, $n = 351$, $p < 0.0005$, Fig. 1B). The same tendency was observed with regard to colonic tumor size, although this difference did not reach statistical significance (2.29 ± 0.29 mm, $n = 54$ vs. 1.78 ± 0.33 mm, $n = 30$, $p = 0.331$, Fig. 1B). In addition, a limited number of $APC^{Min/+}$mPGES-$1^{+/-}$ mice were analysed with regard to tumor multiplicity and size, revealing a phenotype in between $APC^{Min/+}$mPGES-$1^{+/+}$ and $APC^{Min/+}$mPGES-$1^{-/-}$ animals (Fig. 1A–B).

A subset of tumors ($n = 30$) from $APC^{Min/+}$mPGES-$1^{+/+}$ and $APC^{Min/+}$mPGES-$1^{-/-}$ mice were classified histopathologically. In total, 80% of all tumors were classified as high grade tubular adenomas and 20% as invasive adenocarcinomas. These findings did not differ between $APC^{Min/+}$mPGES-$1^{+/+}$ and $APC^{Min/+}$mPGES-$1^{-/-}$ animals (data not shown).

PGE_2 measurements

In accordance with previous reports on human and murine intestinal tumors [15–17], the PGE_2 levels were nearly threefold elevated in $APC^{Min/+}$mPGES-$1^{+/+}$ tumors compared to non-tumor mucosa, but, as expected, similarly low in normal mucosa and tumor tissue of mPGES-1 deleted mice (Fig. 2).

mRNA expression assays

To determine whether the $APC^{Min/+}$mPGES-$1^{-/-}$ individuals exhibited any deviations in the expression of other genes critically involved in PGE_2 signalling, a panel of mRNA expression assays was performed on tumors and corresponding non-tumor mucosa spec-

Table 1
Mean body weight ± SEM (g)

Genotype	$APC^{Min/+}$mPGES-$1^{+/+}$	$APC^{Min/+}$mPGES-$1^{-/-}$	*p* Value[a]
Sex			
Male	34.00 (±0.63) ($n = 12$)	30.14 (±0.67) ($n = 8$)	0.002
Female	26.75 (±0.59) ($n = 8$)	23.25 (±0.63) ($n = 4$)	0.004

[a] Mean body weights were compared with Student's *t*-test.

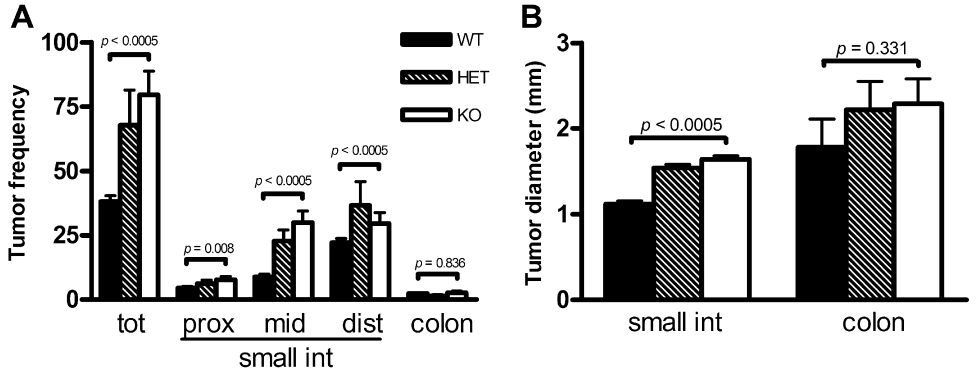

Fig. 1. Tumor multiplicity and size. (A) Mean tumor frequency (±SEM) per mouse among APC$^{Min/+}$mPGES-1$^{+/+}$ (WT, $n = 20$), APC$^{Min/+}$mPGES-1$^{+/-}$ (HET, $n = 4$) and APC$^{Min/+}$mPGES-1$^{-/-}$ (KO, $n = 12$) mice. Tot, total intestine. Prox., Mid., and Dist stand for proximal, middle, and distal third of small intestine, respectively. (B) Mean diameter (±SEM) of small and large intestinal tumors among APC$^{Min/+}$mPGES-1$^{+/+}$, APC$^{Min/+}$mPGES-1$^{+/-}$, and APC$^{Min/+}$mPGES-1$^{-/-}$ animals.

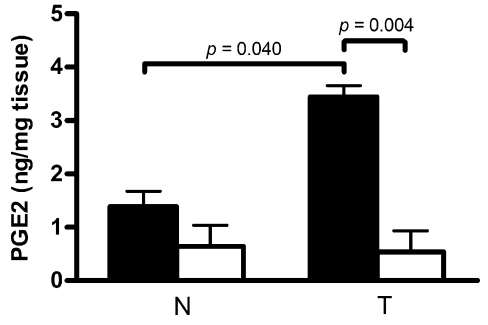

Fig. 2. PGE_2 concentration (mean ± SEM) in tumors and corresponding non-tumor intestinal mucosa, expressed as ng PGE_2 per mg tissue. Black columns = APC$^{Min/+}$mPGES-1$^{+/+}$ ($n = 9$). White columns = APC$^{Min/+}$mPGES-1$^{-/-}$ ($n = 9$). N, non-tumor intestinal mucosa. T, tumor tissue. Error bars = SEM.

imens. As expected, COX-2 and mPGES-1 mRNA expression was induced in APC$^{Min/+}$mPGES-1$^{+/+}$ tumors (Fig. 3A and B). COX-2 was similarly upregulated in APC$^{Min/+}$mPGES-1$^{-/-}$ tumors (Fig. 3A) but as expected, no mPGES-1 mRNA was detectable in any tissue of these mice (Fig. 3B). With regard to the expression of COX-1, mPGES-2, cPGES, 15-PGDH, and prostaglandin E receptors (EP)1–4, no major differences between genotypes were observed (Fig. 3C–J).

Measurment of TxB_2, PGD_2-MOX, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$

In addition to PGE_2–TxA_2, PGD_2, PGI_2, and $PGF_{2\alpha}$ are all derived from PGH_2. To elucidate whether the generation of these bioactive lipids was affected upon mPGES-1 deletion, we determined their concentrations or the concentrations of their corresponding stable metabolites/derivatives ($TxA_2 \rightarrow TxB_2$, $PGI_2 \rightarrow$ 6-keto-$PGF_{1\alpha}$, $PGD_2 \rightarrow$ PGD_2-MOX) in tumors and adjacent mucosa.

Notably, there was a tendency towards higher levels of TxB_2, PGD_2, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$ in tumors of mPGES-1 deficient animals compared to mPGES-1 wild type littermates, being most prominent with regard to TxB_2 and PGD_2 (Fig. 4A–D). A similar, tendency was evident for TxB_2, PGD_2, and 6-keto-$PGF_{1\alpha}$ in non-tumor mucosa of APC$^{Min/+}$mPGES-1$^{-/-}$ compared to APC$^{Min/+}$mPGES-1$^{+/+}$ individuals (Fig. 4A–C).

Discussion

The induced expression of COX-2 with subsequent generation of downstream lipid mediators, including PGE_2, has been considered to be a crucial event in the development of colorectal tumors

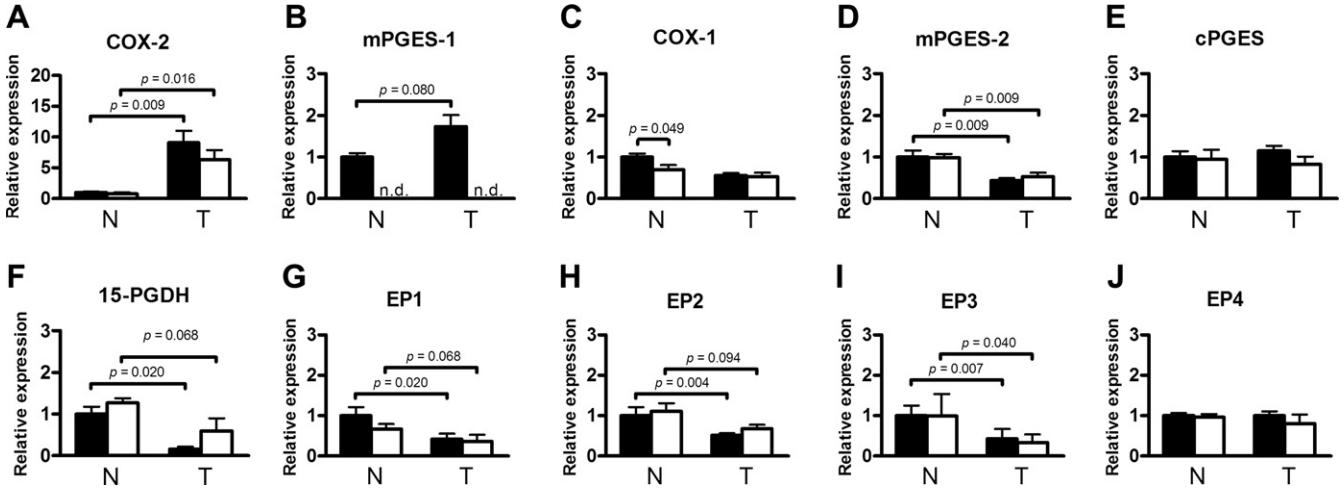

Fig. 3. Relative mRNA expression of COX-2 (A), mPGES-1 (B), COX-1 (C), mPGES-2 (D), cPGES (E), 15-PGDH (F), and EP1-4 (G–J) in tumors and corresponding non-tumor intestinal mucosa of APC$^{Min/+}$mPGES-1$^{+/+}$ and APC$^{Min/+}$mPGES-1$^{-/-}$ mice. mRNA expression was related to endogenous control gene GAPDH and normalised to the average expression in non-tumor intestinal mucosa of APC$^{Min/+}$mPGES-1$^{+/+}$ animals. Black columns = APC$^{Min/+}$mPGES-1$^{+/+}$ ($n = 14$). White columns = APC$^{Min/+}$mPGES-1$^{-/-}$ ($n = 9$). Error bars = SEM.

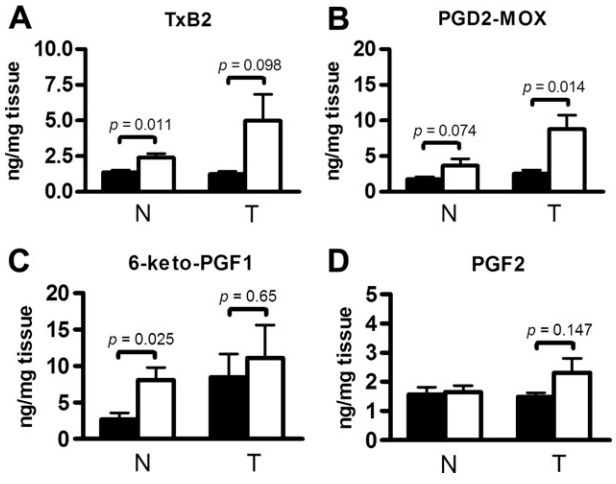

Fig. 4. TxB2 (A), PGD2-MOX (B), 6-keto-$PGF_{1\alpha}$ (C), and $PGF_{2\alpha}$ (D) concentrations (mean ± SEM) in tumors and corresponding non-tumor intestinal mucosa, expressed as ng per mg tissue. Black columns = $APC^{Min/+}mPGES\text{-}1^{+/+}$ ($n = 9$). White columns = $APC^{Min/+}mPGES\text{-}1^{-/-}$ ($n = 9$). N, non-tumor intestinal mucosa. T, tumor tissue. Error bars = SEM.

[1]. Ultimately, the COX-2 metabolite PGH_2 is converted into PGE_2 by mPGES-1, that is upregulated in inflammatory and neoplastic states [11,12,18]. In the present study, $APC^{Min/+}$ mice with homozygous deletion of the mPGES-1 encoding gene (PTGES) were generated to assess the role for mPGES-1 in intestinal carcinogenesis.

Notably, we observed accelerated tumorigenesis among the mPGES-1 deleted animals, which was reflected by the increased size and frequency of intestinal tumors. These findings surprised us, since genetic deletion or pharmacological inhibition of COX-2, thought to be functionally coupled with mPGES-1 [12,18], strongly suppresses tumor development in rodent models of intestinal cancer [19–22]. Accordingly, the usage of both traditional NSAIDs and COX-2 selective inhibitors has been associated with markedly decreased risk of developing colorectal cancer in epidemiological studies (reviewed in [1]).

Though, in the light of severe systemic side effects associated with long term COX targeting therapy, most probably due to the altered balance of non-PGE_2 prostanoids downstream of COX-2/PGH_2, potential ways of selectively targeting PGE_2 generation/signalling have been discussed [1]. In this context, inhibition of mPGES-1 has emerged as an attractive approach. In the present study, we demonstrate that targeting mPGES-1 indeed reduces tumor generation of PGE_2, but, nevertheless, is associated with an increased rather than reduced tumor load.

Since recent studies have indicated that targeting mPGES-1 might not only suppress PGE_2 generation *per se*, but also redirect the metabolism of PGH_2 towards other lipid mediators [23–25], we elucidated this possibility in tumors and non-tumor intestinal mucosa of $APC^{Min/+}mPGES\text{-}1^{+/+}$ and $APC^{Min/+}mPGES\text{-}1^{-/-}$ littermates. Strikingly, we observed a general tendency towards higher concentrations of all non-PGE_2 PGH_2 metabolites in tumors of mPGES-1 deleted animals when compared to mPGES-1 wild type littermates, being most prominent for TxB_2 (stable TxA_2 metabolite) and PGD_2, although 6-keto-$PGF_{1\alpha}$ (stable PGI_2 metabolite) and $PGF_{2\alpha}$ were slightly enhanced as well. The prostanoid profile in the normal intestinal mucosa was, albeit less pronounced than in corresponding tumor tissue, similarly shifted towards enhanced concentrations of TxB_2, PGD_2, and 6-keto-$PGF_{1\alpha}$ among mPGES-1 knockout animals. Importantly, although most focus has been on PGE_2 so far, recent reports suggest these substances to be critically involved in carcinogenesis as well.

First, thromboxane A_2 was shown to promote tumor growth and angiogenesis [2,3], and inhibition of TxA_2 synthase blocked the development of colorectal cancer liver metastases *in vivo* [4]. Second, $PGF_{2\alpha}$ was found to stimulate motility and invasiveness of colorectal cancer cells *in vitro* [5], and PGI_2 was able to promote colon cell survival through interaction with PPARδ receptor [6,7], which promotes intestinal tumorigenesis *in vivo* [8].

With regard to PGD_2 in tumorigenesis, conflicting data are present. Deletion of hematopoietic prostaglandin D synthase (H-PGDS) was shown to accelerate tumor development in $APC^{Min/+}$ mice [26], which was however not the case with regard to colon tumor formation in AOM-treated mice with targeted deletion of the prostaglandin D (DP) receptor[9]. On the other hand, selective inhibition of DP in post-colitis rats reduced, rather than increased, their susceptibility to aberrant crypt foci development [10], implying a tumor promoting role for PGD_2 in colitis associated cancer. These discrepancies might reflect the ability of PGD_2 and its bioactive metabolites to interact with not only the DP receptor, but also the PPARγ receptor which is associated with apoptosis and growth inhibition of colon cancer cells [27].

Taken together, a redirection towards the generation of one or several of these potentially protumorigenic mediators emerges as a possible mechanism whereby targeting mPGES-1 might promote intestinal tumorigenesis. Interestingly, this phenomenon was recently discussed in the pain/hyperalgesia context, where mPGES-1 deletion surprisingly failed to suppress nociceptive behaviour in mice, probably due to metabolic shunting of PGE_2 towards PGD_2, PGI_2, and $PGF_{2\alpha}$ [24], and has previously been reported in gastric mucosa and immune-challenged macrophages as well [23,25].

In summary, we here demonstrate that deletion of the mPGES-1 encoding gene in $APC^{Min/+}$ mice suppresses PGE_2 generation in tumor tissue but, unexpectedly, accelerates intestinal tumorigenesis. A redirected generation of PGE_2 towards other bioactive prostanoids/thromboxanes exerts a possible mechanism and underlines the need for further studies on the roles for these mediators in carcinogenesis *in vivo*. Nevertheless, our findings clearly raise concerns regarding the suitability for mPGES-1 targeting therapy in the treatment or prevention of colorectal cancer.

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