15-Hydroxyprostaglandin dehydrogenase is an *in vivo* suppressor of colon tumorigenesis

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15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is a prostaglandin-degrading enzyme that is highly expressed in normal colon mucosa but is ubiquitously lost in human colon cancers. Herein, we demonstrate that 15-PGDH is active *in vivo* as a highly potent suppressor of colon neoplasia development and acts in the colon as a required physiologic antagonist of the prostaglandin-synthesizing activity of the cyclooxygenase 2 (COX-2) oncogene. We first show that 15-PGDH gene knockout induces a marked 7.6-fold increase in colon tumors arising in the Min (multiple intestinal neoplasia) mouse model. Furthermore, 15-PGDH gene knockout abrogates the normal resistance of C57BL/6J mice to colon tumor induction by the carcinogen azoxymethane (AOM), conferring susceptibility to AOM-induced adenomas and carcinomas *in situ*. Susceptibility to AOM-induced tumorigenesis is mediated by a marked induction of dysplasia, proliferation, and cyclin D1 expression throughout microscopic aberrant crypt foci arising in 15-PGDH null colons and is concomitant with a doubling of prostaglandin E_2 in 15-PGDH null colonic mucosa. A parallel role for 15-PGDH loss in promoting the earliest steps of colon neoplasia in humans is supported by our finding of a universal loss of 15-PGDH expression in microscopic colon adenomas recovered from patients with familial adenomatous polyposis, including adenomas as small as a single crypt. These models thus delineate the *in vivo* significance of 15-PGDH-mediated negative regulation of the COX-2 pathway and moreover reveal the particular importance of 15-PGDH in opposing the neoplastic progression of colonic aberrant crypt foci.

colon cancer | prostaglandin E_2

The first and rate-limiting step in the inactivation and degradation of prostaglandins is catalyzed by the enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (1). Studies by our group and by others have demonstrated that 15-PGDH is highly expressed by normal colonic epithelial cells residing in the luminal regions of colonic crypts but that transcription of 15-PGDH mRNA is ubiquitously lost in colon cancers (2, 3). These findings have suggested the hypothesis that 15-PGDH could be a candidate tumor suppressor gene (2, 3) that might, in the normal colon, act to antagonize the prostaglandin-generating activity of the cyclooxygenase 2 (COX-2) oncogene (4). Transcriptional up-regulation of COX-2 is thought to contribute to the genesis of up to 85% of all human colon cancers (4), with the oncogenic activity of COX-2 having been demonstrated in multiple different *in vivo* models (5–7) and by the activity of COX-2-inhibitory drugs in shrinking premalignant human colonic adenomas (4, 8). Although negative regulation of the COX-2 pathway by 15-PGDH could thus be of clear potential significance to colon carcinogenesis, the hypothesized tumor suppressor activity of 15-PGDH has thus far not been tested *in vivo*. We therefore embarked on a series of studies designed to test the *in vivo* potency of 15-PGDH as a colon tumor suppressor by using the 15-PGDH knockout mouse as an assay system. We additionally used this model to delineate the earliest stages of colon neoplasia for which presence or absence of 15-PGDH suppressor activity would be determinative.

Results

15-PGDH Suppression of Azoxymethane (AOM)-Induced Colon Tumors. To first investigate the potential *in vivo* activity of 15-PGDH as a suppressor of colonic neoplasia, we bred the 15-PGDH null knockout allele (9) onto the C57BL/6J mouse strain, which has been well characterized as being highly resistant to colon tumor induction by the carcinogen AOM (10). Intercrossing mice heterozygous for the 15-PGDH null allele produced litters including mice with 15-PGDH genotypes that were +/+, +/−, and −/−. As expected, 15-PGDH protein was totally absent in colons of mice having the −/− genotype (Fig. 1*a*). Littermates of all genotypes were identically treated with six i.p. doses of AOM. As reported in ref. 10, wild-type 15-PGDH +/+ C57BL/6J mice proved highly resistant to AOM treatment, with no induction of any tumors in these mice. In contrast, in 15-PGDH −/− mice, loss of 15-PGDH conferred marked susceptibility to colon tumor induction, with 0.75 ± 0.18 (results are given as mean ± SEM) tumors arising per mouse (Poisson regression with contrasts, $P < 0.0001$) (Fig. 1 *a* and *b*). On histopathology review, half of the tumors arising in the 15-PGDH −/− mice were tubular adenomas (Fig. 1 *a* and *c*), whereas the other half of tumors had further progressed to carcinomas *in situ*, as assessed by the finding of high-grade dysplasia (Fig. 1 *a* and *d*). Intriguingly, loss of even one 15-PGDH allele appeared to partially sensitize mice to colon tumor development, with induction of 0.15 ± 0.08 tumors per 15-PGDH +/− mouse ($P < 0.024$).

15-PGDH Suppression of Colon Tumorigenesis in the Min Mouse. It remained potentially possible that the susceptibility of the 15-PGDH −/− mice to colon tumor induction might be related to an indirect effect of 15-PGDH loss on the metabolism of the AOM carcinogen. Therefore, we tested the effect of 15-PGDH gene knockout in a carcinogen-independent model, that of the well-studied Min (multiple intestinal neoplasia) mouse that

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Abbreviations: 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; COX-2, cyclooxygenase 2 [formally referred to as prostaglandin-endoperoxide synthase 2 (PTGS2)]; AOM, azoxymethane; FAP, familial adenomatous polyposis; ACF, aberrant crypt foci; PGE_2, prostaglandin E_2.

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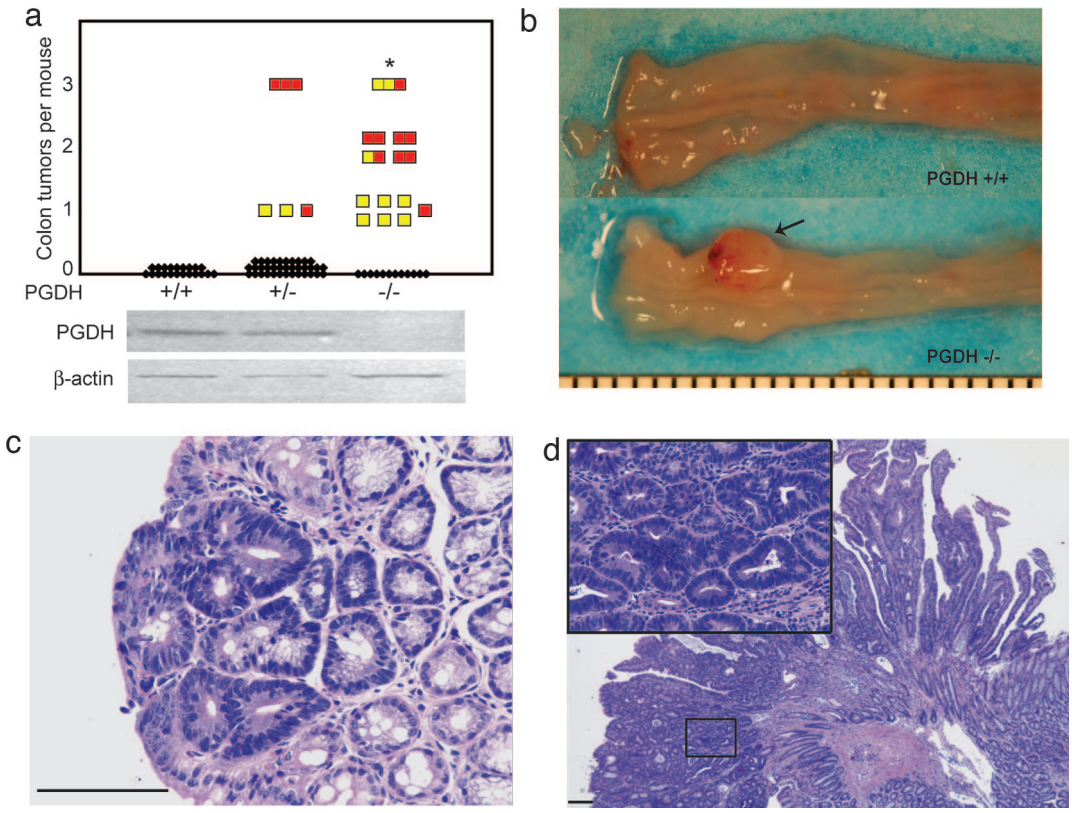

Fig. 1. Colon tumor induction by AOM. (*a*) Tumor development in 15-PGDH $+/+$ ($n = 21$), $+/-$ ($n = 40$), and $-/-$ ($n = 24$) C57BL/6J mice. Diamonds indicate mice without tumors. Boxes designate mice with colon tumors, with box size proportional to tumor number. Yellow fill designates tubular adenomas, and red fill designates tumors with high-grade dysplasia (also termed "carcinoma *in situ*"). *, $P < 0.0001$ for increased total colon tumors, $P < 0.0008$ for increased carcinoma *in situ* tumors, $-/-$ vs. $+/+$ mice. Western blot assay of colonic 15-PGDH and actin are shown below each of the mice genotypes. (*b*) Gross morphology of a tumor (arrow) in 15-PGDH$-/-$ mouse colon (*Lower*) compared with colon of a 15-PGDH$+/+$ mouse (*Upper*). (*c* and *d*) Representative histopathology of AOM-induced tumors. (*c*) Adenomatous polyp. (Scale bar, 100 μm.) (*d*) High-grade dysplasia (equivalently termed carcinoma *in situ*). (Scale bar, 200 μm for low-power field, with *Inset* magnified to same scale as *c*.)

carries a germline mutant copy of the adenomatous polyposis coli (APC) colon cancer suppressor gene (11). In humans, germline carriage of a mutant APC allele gives rise to familial adenomatous polyposis (FAP), a syndrome in which affected individuals by the fourth decade of life develop hundreds of colonic adenomas and ultimately colon cancer (12–14). In Min mice, germline mutant APC induces a similarly dramatic intestinal neoplasia phenotype, with typically 60 or more intestinal adenomas developing per mouse (11). However, adenomas in the Min mouse typically develop almost exclusively within the small intestine and rarely involve the colon (11). In this study, the 15-PGDH null allele was bred into the Min mouse, on a C57BL/6J background, and the mice were intercrossed. Littermates were obtained that demonstrated compound genotypes of $APC^{+/Min}PGDH^{+/+}$, $APC^{+/Min}PGDH^{+/-}$, and $APC^{+/Min}PGDH^{-/-}$. $APC^{+/Min}PGDH^{+/+}$ and $APC^{+/Min}PGDH^{+/-}$ mice showed essentially identical phenotypes (Table 1, which is published as supporting information on the PNAS web site) and together developed on average 58.8 ± 6.1 small intestinal tumors and 1.0 ± 0.3 colon tumors per mouse (Fig. 2 *a* and *b* and Table 1). In contrast, $APC^{+/Min}PGDH^{-/-}$ mice that were nullizygous for 15-PGDH demonstrated a nearly 8-fold increase in colon tumor development (Fig. 2 *a*, *c*, and *d*), developing an average 7.6 ± 2.4 colon adenomas per mouse (Poisson regression with contrasts, $P < 0.0001$). $APC^{+/Min}PGDH^{-/-}$ mice demonstrated a more modest 52% increase in small intestinal adenomas (Fig. 2*b*), developing an average of 89.4 ± 6.6 tumors per mouse ($P < 0.0001$). Thus, loss of 15-PGDH markedly increases susceptibility of the mouse colon to developing epithelial tumors, irrespective of whether these tumors are initiated by AOM or by a germline mutant APC allele.

15-PGDH Regulation of Dysplasia in Colonic Aberrant Crypt Foci (ACF). To explore the mechanism by which loss of 15-PGDH confers increased susceptibility to colon neoplasia, we first determined the effect of 15-PGDH knockout on levels of colonic prostaglandin E_2 (PGE_2), the predominant prostaglandin of the colonic mucosa, whose activity has been implicated in intestinal tumor development (6). 15-PGDH null mice demonstrated a doubling in colonic mucosal PGE_2, with an average of 4.90 ± 0.62 ng/mg of protein in the $-/-$ mice vs. 2.51 ± 0.33 ng/mg of protein in the $+/+$ mice (Student's *t* test, $P = 0.004$). To investigate the consequences of this increased colonic mucosal PGE_2, we first examined normal colonic mucosa from 15-PGDH $+/+$ vs. $-/-$ mice, both before and after treatment with AOM. However, no differences were seen between these normal tissues with respect to immunostaining for markers of proliferation (Ki-67), apoptosis (TUNEL), or signaling targets (nuclear β-catenin, cyclin D1, phospho-AKT, and phospho-ERK), all of which are known to be modulated by PGE_2 treatment of neoplastic intestinal cells (data not shown) (15–19). We therefore hypothesized that elevated mucosal PGE_2, rather than altering the normal colonic mucosa, might favor the neoplastic progression of the initiated colonic epithelial cell. To explore this hypothesis, we examined microscopic ACF, which are the earliest neoplastic precursor lesions initiated by the AOM car-

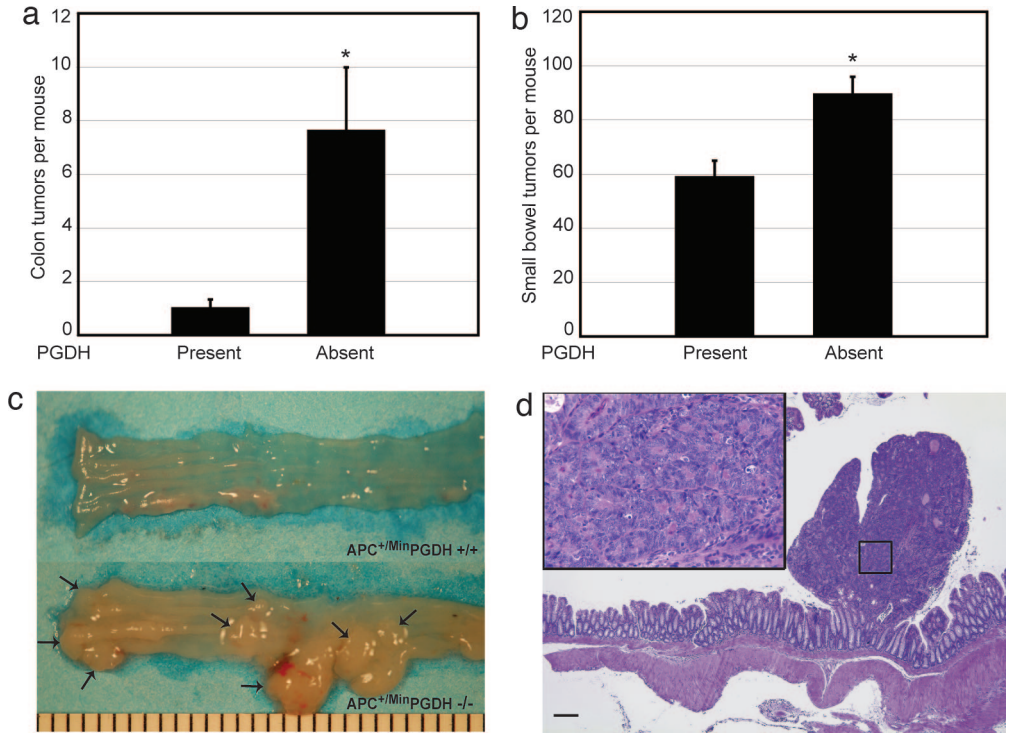

Fig. 2. Tumor induction in the $APC^{+/Min}$ mouse. (*a* and *b*) Shown are the number of tumors per mouse in a combined cohort of $APC^{+/Min}PGDH^{+/+}$ and $APC^{+/Min}PGDH^{+/-}$ mice (total $n = 21$) in which 15-PGDH is present (Present) vs. $APC^{+/Min}PGDH^{-/-}$ mice ($n = 13$) in which 15-PGDH is absent (Absent). (*a*) Colon tumors. *, $P < 0.0001$ for increased colon tumors in 15-PGDH-absent mice. (*b*) Small intestinal tumors. *, $P < 0.0001$ for increased small intestinal tumors in 15-PGDH-absent mice. (*c*) Gross morphology of tumors (arrows) in a representative $APC^{+/Min}PGDH^{-/-}$ mouse colon (*Lower*) compared with colon of an $APC^{+/Min}PGDH^{+/+}$ mouse (*Upper*). (*d*) Representative histopathology of a colon tumor from an $APC^{+/Min}PGDH^{-/-}$ mouse. (Scale bar, 200 μm, with *Inset* as in Fig. 1*d*.)

cinogen (20), and which we compared between 15-PGDH +/+ vs. −/− colons (Fig. 3*c*). 15-PGDH −/− mice did demonstrate a modest 41% increase in total AOM-induced ACF vs. the +/+ mice (14.8 ± 2.1 vs. 10.5 ± 1.5, respectively; Poisson regression with contrasts, $P < 0.007$) (Fig. 3*a*). More substantially, −/− mice exhibited a 4-fold elevation in numbers of ACF that were able to attain a size of four crypts or greater (2.9 ± 0.7 vs. 0.7 ± 0.3 large ACF per colon, +/+ vs. −/− mice, respectively; $P < 0.0001$) (Fig. 3*b*), thereby suggesting an increased propensity for progression of the ACF arising in the 15-PGDH −/− mice. Further histopathology review indeed demonstrated that ACF in the −/− mice represent a clearly progressed and more aggressive class of lesions (21, 22) (Fig. 3 *d–g*). Thus, <10% of 15-PGDH +/+ mouse ACF exhibited any of the following: (*i*) histologic features of moderate or severe dysplasia (Fig. 3 *d* and *e*), (*ii*) positive staining for nuclear cyclin D1 (Fig. 3 *d* and *f*), or (*iii*) expansion of the Ki-67 positive zone of proliferative cells above the lower one-half of the crypts (Fig. 3 *d* and *g*). In marked contrast, 15-PGDH −/− mouse ACF commonly demonstrated (*i*) moderate to severe dysplasia (56 ± 10%) (Fig. 3 *d* and *e*), (*ii*) positive staining for nuclear cyclin D1 (64 ± 10%) (Fig. 3 *d* and *f*), and (*iii*) extension of the Ki-67 positive zone of cellular proliferation reaching to the luminal surface of the crypts (83 ± 8%) (Fig. 3 *d* and *g*). Each of these differences was highly statistically significant (Fisher's exact test for −/− vs. +/+ mice, $P < 0.003$ for increased dysplasia, $P < 0.0008$ for increased nuclear cyclin D1, and $P < 0.0001$ for increased Ki-67). Moreover, expansion of the Ki-67 positive proliferative zone was demonstrated in every ACF studied that had independently been graded as having moderate to severe dysplasia, with 80% of these lesions also staining positive for nuclear cyclin D1. Thus, the coordinate induction of moderate to severe dysplasia, expression of nuclear cyclin D1, and Ki-67 expression extending to the luminal crypt surface delineates an early neoplastic cell population whose outgrowth is directly fostered by loss of 15-PGDH and whose emergence in the 15-PGDH −/− mice directly translates into the further development of frank colonic adenomas and carcinoma *in situ* tumors.

15-PGDH Loss in Microscopic Human Colonic Neoplasias. Previous studies by our group and others have demonstrated that 15-PGDH expression is ubiquitously lost in human colon cancers (2, 3). To determine whether loss of 15-PGDH in humans might, as in the mouse, also be important in promoting the early steps of colon neoplasia development, we examined material from the resected colons of nine human patients with FAP. As expected, immunostaining was positive for the expression of 15-PGDH protein in 41 of 41 histologically normal mucosal samples examined from these patients (Fig. 4*a*). In marked contrast, 15-PGDH staining was completely absent in 118 of the 126 FAP colon adenomas that were examined (Fisher's exact test, $P < 0.0001$), including being undetectable in 28 of 31 adenomas that were <1 mm but more than eight crypts in size ($P < 0.0001$), and including being undetectable in 26 of 28 adenomas that were less than eight crypts in size ($P < 0.0001$) (Fig. 4*a*). Indeed, loss of 15-PGDH was demonstrable in the earliest lesion possible, that of adenomatous conversion involving only a single crypt (Fig. 4*b*). Thus, in humans, inactivation of 15-PGDH appears to be closely linked with the earliest steps in the development of colonic dysplasia.

Discussion

These findings demonstrate that 15-PGDH is a potent *in vivo* suppressor of colon neoplasia development whose inactivation promotes development of colon neoplasias, both in mice and in humans. We find that 15-PGDH normally acts as a physiologic

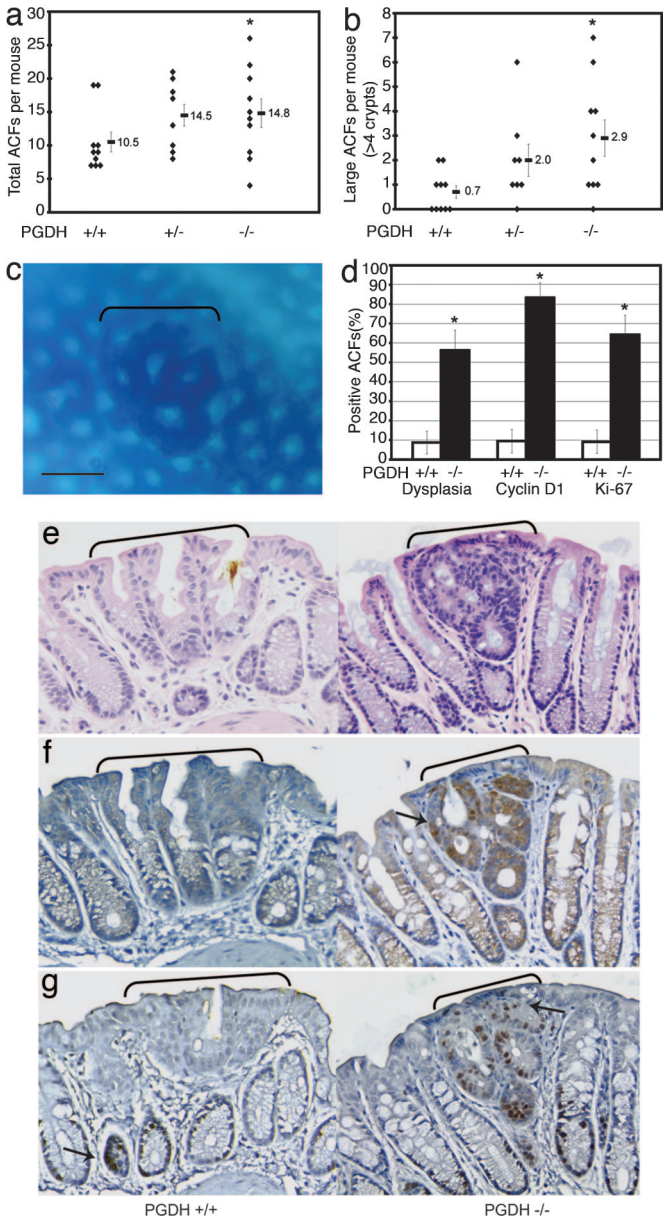

Fig. 3. ACF induction by AOM. (*a* and *b*) Diamonds indicate numbers of ACF observed for each colon examined from 15-PGDH +/+ ($n = 10$), +/− ($n = 8$), and −/− ($n = 10$) mice. Horizontal bars designate group means. Error bars denote SEMs. (*a*) Total ACF. *, $P < 0.007$ for increase in ACF in 15-PGDH −/− mice. (*b*) Large ACF (four or more crypts). *, $P < 0.0001$ for increase in large ACF in 15-PGDH−/− mice. (*c*) Methylene blue-stained ACF (bracketed) encompassing four crypts. (Scale bar, 100 μm.) (*d*) Percentage of ACF from 15-PGDH +/+ mice (open bars) vs. 15-PGDH −/− mice (filled bars) exhibiting moderate to severe dysplasia (+/+, $n = 23$ ACF from eight mice; −/−, $n = 16$ ACF from nine mice), nuclear cyclin D1 (+/+, $n = 22$ ACF from eight mice; −/−, $n = 14$ ACF from eight mice), and Ki-67 staining of the upper half of the crypt (+/+, $n = 21$ ACF from eight mice; −/−, $n = 12$ ACF from six mice). *, $P < 0.003$ for increased dysplasia, $P < 0.0008$ for increased cyclin D1, and $P < 0.0001$ for increased Ki-67. (*e–g*) A representative aberrant crypt focus (bracketed) from a 15-PGDH +/+ (*Left*) vs. a −/− (*Right*) mouse, with serial sections stained for histology (e), cyclin D1 (*f*), and Ki-67 (*g*). Arrows indicate regions of positive staining.

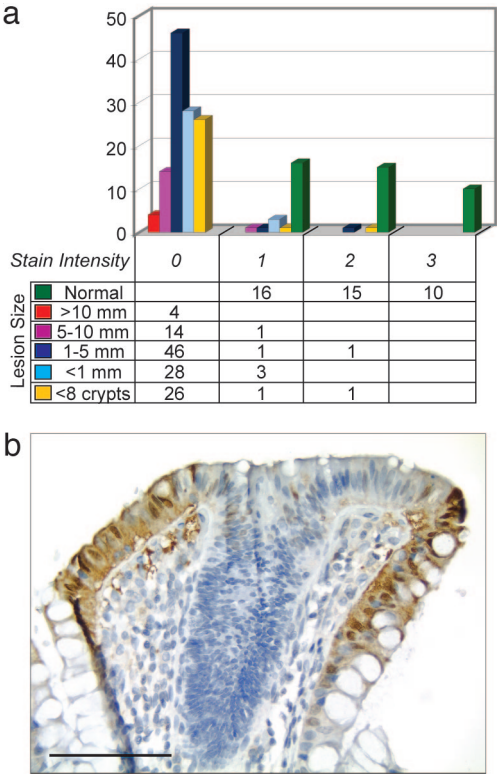

Fig. 4. 15-PGDH loss in FAP. (*a*) Graphical display of 15-PGDH immunostaining intensity (0 to 3+) in nine FAP patients providing 41 normal colonic mucosal samples (green bars) and 126 colon adenomas of sizes from >10 mm to fewer than eight crypts, with additional bar colors denoting adenoma lesions of different size classes. Bar heights denote the number of samples at each staining intensity level within each of the groups of different-sized adenomas, with sample numbers also tabulated beneath each grouping. (*b*) Photomicrograph demonstrating loss of 15-PGDH immunostaining in a single-crypt-sized adenoma vs. presence of 15-PGDH expression in surrounding normal epithelium. (Scale bar, 100 μm.)

negative regulator of prostaglandin levels in the gut. In contrast, inactivation of 15-PGDH is a pathophysiologic event that leads to an increase in colonic mucosal PGE_2 and that promotes the progression of initiated colonic epithelial cells, first to microscopic dysplasias and then to macroscopic tumors. The marked increase in colon tumor susceptibility that is concomitant with the doubling of colonic mucosal PGE_2 suggests both the biological potency of this prostaglandin and the importance of tight physiologic regulation of PGE_2 levels in the gut mucosa. Although theoretically possible, we found no suggestion that increased PGE_2 was converted into $PGF_2\alpha$, another potentially mitogenic prostaglandin, inasmuch as colon mucosal $PGF_2\alpha$ ranged between only 10–20% of the levels of PGE_2 in both 15-PGDH wild-type and knockout mice (data not shown). A potential alternative active prostanoid, TXA_2, is not a substrate of 15-PGDH (1) and so is not anticipated to be modulated in the 15-PGDH knockout mouse. Supporting the interpretation that the doubling of PGE_2 is the mediator of colon tumor susceptibility in the 15-PGDH knockout mouse is the finding that direct administration of PGE_2 to Min mice at doses that only double intestinal PGE_2 levels also similarly increases colon and small intestinal tumor numbers (16).

Our present findings thus further highlight the importance of prostaglandin up-regulation in colon neoplasia pathogenesis, demonstrating that colon neoplasms doubly target this pathway, with neoplasia development driven not only by induction of expression of the COX-2 oncogene (4, 5) but also by concomitant inactivation of expression of the 15-PGDH tumor suppressor gene. Recent attempts to decrease the risk of developing colon cancer by using drugs that inhibit COX-2 activity have proven problematic due to unfavorable cardiovascular side effects associated with these com-

pounds (23, 24). However, we and others have demonstrated that 15-PGDH expression can be reactivated in certain colon cancer cell lines either by restoring TGF-β signaling or by inhibiting EGF-R signaling (2, 3). We accordingly hypothesize that identifying compounds able to act in the gut to more generally reinduce 15-PGDH expression among early neoplastic cells could provide alternative and targeted agents with potential efficacy in colon neoplasia prevention.

Materials and Methods

Human Tissues. Colon tissues were collected under an Institutional Review Board-approved protocol at University Hospitals of Cleveland.

Mice Genotyping. Mice studies were conducted in the Case Animal Resource Center under a protocol approved by the Institutional Animal Care and Use Committee. Genotyping of wild-type and 15-PGDH knockout alleles was done as described in ref. 9. Genotyping of wild-type and Min APC alleles was done as per The Jackson Laboratory web site (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype = protocol&protocoLid = 529).

Min Mouse Studies. Litters were selected that each included mice of differing 15-PGDH genotypes. Complete litters were killed for analysis when signs of severe health impairment were noted in any of the littermates.

AOM Treatment. Six- to 12-week-old mice were injected i.p. once weekly for 6 weeks with 10 mg/kg AOM (10) (Sigma Chemical Co., St. Louis, MO). Mice were killed 24 weeks after the last AOM injection.

Intestinal Tumor Counts. Immediately after killing of the mice, the small bowels and colons were opened longitudinally, rinsed with ice-cold PBS, and examined under a dissecting microscope to identify all tumors. Tumors were resected, fixed in 10% neutral buffered formalin, and paraffin-embedded for histologic examination.

ACF Analysis. ACF were visualized and counted by examination of methylene blue-stained mouse colons under a light microscope (20). ACF locations were marked with tissue ink (Bradley Products, Inc., Bloomington, MN), after which the ACF and surrounding tissue were excised, fixed in 10% neutral buffered formalin, paraffin-embedded, and sectioned vertical to the axis of the colonic crypts. ACF were located by using the surface ink mark and then examined histologically after staining with hematoxylin and eosin.

Ki-67 and Cyclin D1 Immunostaining. Ki-67 was visualized by staining with rat anti-murine Ki-67 monoclonal M7249 (Dako, Carpenteria, CA). Cyclin D1 was visualized by staining with rabbit anti-cyclin D1 antibody RB-9041-R7 (Lab Vision, Inc., Fremont, CA).

15-PGDH Western Blot Analysis and Immunohistochemistry. Western blotting and immunohistochemistry for 15-PGDH were performed by using a monoclonal anti-15-PGDH antibody raised in our laboratory and used in accordance with our previous protocols for 15-PGDH immunodetection (2).

PGE_2 Analysis. PGE_2 was extracted from frozen samples of mouse colon mucosa and quantitated by reverse-phase liquid chromatography electrospray ionization mass spectrometry relative to a deuterated PGE_2 internal standard, following a modification of our published methods (25). Results were expressed as nanograms of PGE_2 per milligram of protein.

Further details regarding these methods are provided in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

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