Up-regulation of CYP26A1 in Adenomatous Polyposis Coli–Deficient Vertebrates via a WNT-Dependent Mechanism: Implications for Intestinal Cell Differentiation and Colon Tumor Development

Dawne N. Shelton,[1,3] Imelda T. Sandoval,[2,3] Annie Eisinger,[1,3] Stephanie Chidester,[3] Anokha Ratnayake,[2] Chris M. Ireland,[2] and David A. Jones[1,2,3]

Departments of [1]Oncological Sciences and [2]Medicinal Chemistry and the [3]Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah

Abstract

Mutations in the *adenomatous polyposis coli* (*APC*) tumor suppressor gene seem to underlie the initiation of many colorectal carcinomas. Loss of APC function results in accumulation of β-catenin and activation of β-catenin/TCF–dependent transcription. Recent studies have implicated APC in controlling retinoic acid biosynthesis during normal intestinal development through a WNT-independent mechanism. Paradoxically, however, previous studies found that dietary supplementation of Apc^{MIN} mice with retinoic acid failed to abrogate adenoma formation. While investigating the above finding, we found that expression of *CYP26A1*, a major retinoic acid catabolic enzyme, was up-regulated in Apc^{MIN} mouse adenomas, human FAP adenomas, human sporadic colon carcinomas, and in the intestine of apc^{mcr} mutant zebrafish embryos. Mechanistically, *cyp26a1* induction following *apc* mutation is dependent on WNT signaling as antisense morpholino knockdown of *tcf4* or injection of a dnLEF construct into apc^{mcr} mutant zebrafish suppressed expression of *cyp26a1* along with known WNT target genes. In addition, injection of stabilized *β-catenin* or *dnGSK3β* into wild-type embryos induced *cyp26a1* expression. Genetic knockdown or pharmacologic inhibition of cyp26a1 in apc^{mcr} mutant zebrafish embryos rescued gut differentiation defects such as expression of intestinal fatty acid–binding protein and pancreatic trypsin. These findings support a novel role for APC in balancing retinoic acid biosynthesis and catabolism through WNT-independent and WNT-dependent mechanisms. (Cancer Res 2006; 66(15): 7571-7)

Introduction

Retinoic acid is derived from dietary retinol (vitamin A) and plays well-characterized roles in determining cell fates and promoting epithelial differentiation (1, 2). The actions of retinoic acid are, therefore, tightly regulated in both temporal and tissue-specific manners. To achieve this tight regulation, the production of retinoic acid is often carefully balanced by its degradation. Alcohol dehydrogenases and short-chain alcohol dehydrogenases initiate the biosynthesis of retinoic acid by converting retinol into retinaldehyde. Further oxidation of retinaldehyde by retinaldehyde dehydrogenases completes the synthesis of retinoic acid (3). The cellular effects of retinoic acid are mediated through the binding of retinoic acid to specific nuclear receptors (retinoic acid receptors and retinoid X receptors) that serve to activate or repress specific genes required for cell fate and differentiation decisions (4).

The production of retinoic acid in tissues is opposed by its degradation through the actions of specific cytochrome P450 enzymes. CYP26A1 has been implicated in restricting the diffusion of retinoic acid in several model systems, including chick, mouse, and zebrafish (5–8). For example, Cyp26 expression seems to permit cell proliferation and expansion in the leading edge of the developing mouse limb bud by counteracting the production of retinoic acid by the proximal trunk. This allows for coordinately regulated differentiation and proliferation (9). Similarly, Cyp26a1 is necessary for establishment of the anterior-posterior axis. Mice homozygous for mutant *Cyp26a1* exhibit truncations of the posterior axis, including the hindgut, and die *in utero* (10). Genetic suppression of this lethality and posterior axis truncation was recently shown by Niederreither et al. (8, 11) through a cross of $Cyp26a1^{-/-}$ and $Raldh2^{+/-}$ mice, which led to a reduction of retinoic acid production. The regulation of *CYP26A1* expression is not fully understood but is likely due to both basal level regulation of the *CYP26A1* promoter, as well as an inducible aspect in the form of a retinoic acid response element (12).

The differentiation properties of retinoic acid have made it attractive as a potential therapeutic for the treatment of a variety of cancers. Its usefulness, however, seems limited in this context because a number of studies suggest that CYP26-mediated destruction of retinoic acid may reduce its efficacy *in vivo*. For example, Lopez-Berestein et al. (13, 14) have found that retinoic acid treatment of acute promyelocytic leukemia patients leads to increased metabolism and clearing of retinoic acid by CYP26A1 in intestinal, endothelial, and liver tissues. In addition, colon carcinomas and colon carcinoma cell lines have proven unresponsive to retinoic acid treatment (15–17). Finally, recent literature has emerged suggesting that mice bearing mutations in the *Apc* tumor suppressor gene do not show a suppression of adenoma formation when fed with a daily dietary supplement of *all-trans* retinoic acid (ATRA; ref. 18).

The lack of responsiveness of Apc^{MIN} mice to retinoic acid is surprising given recent evidence showing that adenomatous polyposis coli (APC) controls the production of retinoic acid and that this control is essential for normal intestinal differentiation in zebrafish. Specifically, Jette et al. (19) showed that a human retinol dehydrogenase (RDH), *DHRS9*, is lost in colon tumors compared with normal colon tissue. Moreover, this enzyme was induced upon reintroduction of wild-type APC into colon cancer cell lines, thereby linking APC function with retinoic acid biosynthesis for the first time. Extending these findings, Nadauld et al. (20) showed that

Requests for reprints: David A. Jones, Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Salt Lake City, UT 84112. Phone: 801-585-6107; Fax: 801-585-0900; E-mail: david.jones@hci.utah.edu.

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knockdown of *rdh1, rdh1l*, or *APC* by antisense morpholino oligonucleotide injection led to a loss of terminal differentiation in the developing zebrafish gut, as well as other retinoic acid–deficient phenotypes, such as pericardial edema, lack of fin and jaw development, and loss of pancreatic differentiation. Treatment of apc-deficient zebrafish embryos (apc^{mcr}) with ATRA partially rescued the expression of the gut differentiation markers, such as intestinal fatty acid–binding protein (i-fabp) and pancreatic trypsin.

Interestingly, APC control of retinoic acid production seems independent of the canonical model for APC function (19). In the canonical model, APC negatively regulates WNT signaling by helping to target the transcriptional coactivator β-catenin for ubiquitin-mediated proteolysis. Mutations in *APC*, therefore, lead to accumulation of β-catenin and subsequent activation of β-catenin/LEF transcriptional complexes. These complexes are thought to stimulate intestinal cell proliferation through the direct targeting of proliferation genes like *c-myc* (21) and *BMP4* (22). Although our previous work showed significant rescue of *i-fabp* in apc^{mcr} mutant fish by retinoic acid treatment, this rescue was incomplete. This observation, combined with the lack of effect of retinoic acid in the Apc^{MIN} mouse, prompted us to examine the responsiveness of APC-deficient tissues to exogenous retinoic acid. Here, we show that murine, human, and zebrafish tissues harboring mutated *APC* exhibit increased levels of *CYP26A1* and that this increased expression results from activation of WNT signaling. Inhibition of cyp26a1 enzymatic activity or morpholino knockdown of *cyp26a1* restored intestinal differentiation in apc^{mcr} mutant zebrafish. Based on these findings, we propose that APC coordinately regulates retinoic acid production and catabolism through independent pathways.

Materials and Methods

Zebrafish stocks and embryo culture. Wild-type and apc^{mcr} mutant *Danio rerio* (zebrafish) were maintained on a 14:10-hour light/dark cycle. Fertilized embryos were collected following natural spawning and allowed to develop at 28.5°C. Control and experimental embryos were raised in 0.003% phenylthiourea to inhibit pigment formation. Drs. Anna-Pavlina Haramis and Hans Clevers (Hubrecht Laboratorium, NIOB/KNAW, Center for Biomedical Genetics, CT, Utrecht, The Netherlands) kindly provided apc^{mcr} mutant zebrafish used in this study.

Whole-mount *in situ* hybridizations. Zebrafish embryos were dechorionated, fixed in sucrose-buffered 4% paraformaldehyde, rinsed in PBS, dehydrated in methanol, and stored at −20°C. Digoxigenin-labeled riboprobes for *cyp26a1* and *i-fabp* were generated as described previously (23). Both riboprobes were generated by linearization of pCRII (Invitrogen, Carlsbad, CA) containing *cyp26a1* or *i-fabp* cDNA followed by *in vitro* transcription with SP6 RNA polymerase (Roche Applied Science, Indianapolis, IN). Whole-mount *in situ* hybridizations were carried out as described previously (23). Embryos were fixed in 4% paraformaldehyde for 1 hour at room temperature, cleared in methanol overnight, and then cleared in 70% glycerol and PBS and photographed using an Olympus DP12 digital camera.

Quantitative reverse transcription-PCR. Single-stranded cDNA was synthesized from 2 to 3 μg of total RNA using Superscript III (Invitrogen). PCR was done with 30 ng cDNA using the Roche LightCycler instrument and software, version 3.5 (Roche Diagnostics, Indianapolis, IN). Primers were as follows: z.f.cyp26a1, forward, 5′-ACGAGCAAGAACTGGTGGAA-3′; reverse, 5′-GGGCGTCTTTGTATTTCTGCT-3′; z.f.mmp13, forward, 5′-CTTC-TGGCGCAGTTATCCTC-3′; reverse, 5′-TGTACTGGAAGGCTGCAGTG-3′, m.m.Cyp26a1, forward, 5′-GATAAAGAGCAAGGGCTTACT-3′, reverse, 5′-AAATAACATTCCAGCCCTTGG-3′, h.s.CYP26A1, forward, 5′-GCAGGAAA-TACGGCTTCATC-3′, reverse, 5′-CCCGCATAATCACCTTCTTG-3′. PCR was done in triplicate using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science). PCR conditions were as follows: 40 cycles of amplification with 20 seconds of denaturation at 95°C and 20 seconds of annealing at 60°C for cyp26a1 and 35 cycles of amplification with 20 seconds of denaturation at 95°C and 20 seconds of annealing at 55°C for all others. A template-free negative control was included in each experiment. Statistical analyses were done using GraphPad Prism (version 4.03).

Morpholino microinjections. Morpholino oligonucleotides were obtained from Gene Tools LLC (Philomath, OR). The *apc* splice-blocking morpholino (5′-TAGCATACTCTACCTGTGCTCTTCG-3′), z.f.*tcf4* splice-blocking morpholino (5′-CTTATTTGTCACTTACCTCGGAATC-3′), and z.f.*cyp26a1* splice-blocking morpholino (5′-CCCTCAAACCCTGCCGAT-CAAAAAT-3′) were solubilized to 1 mmol/L in 1× Danieau buffer. For microinjections, 1.5 nL of 0.5 mmol/L *apc* antisense morpholino, 1 nL of 0.25 mmol/L *tcf4* morpholino, or 1 nL of 0.0625 mmol/L *cyp26a1* morpholino was injected into wild-type or apc^{mcr} mutant embryos at the one- to two-cell stages (23).

RNA microinjections. For RNA injection experiments, full-length human *β-catenin* or *dnGSK3β* mRNA was synthesized from a linearized pCRII construct using mMessage mMachine (Ambion, Austin, TX) according to the protocol of the manufacturer. Full-length *β-catenin* or *dnGSK3β* mRNAs were injected into embryos at the one-cell stage.

Retinoic acid and drug treatments. To rescue apc^{mcr} mutants by application of retinoic acid, the embryos were incubated in 900 nmol/L ATRA in Me_2SO at 70% epiboly for 45 minutes. in the dark. The embryos were then washed in embryo water thrice. Retinoic acid treatments (100 nmol/L) were repeated every 24 hours for 45 minutes. Control embryos were treated over these periods with an equal volume of Me_2SO (24). Retinoic acid metabolism blocking agent (RAMBA) treatments were the same, with the exception of 2- and 1-day applications, where RAMBA (vn/14-1) was applied at 900 nmol/L at 48 hpf and 100 nmol/L at 72 hpf for 2-day application, or 900 nmol/L at 72 hpf for 1-day application. RAMBA was synthesized as previously reported (23, 25).

Histologic analyses. Embryos were mounted in 3% low-melting-point agarose and then processed and embedded using the Polysciences Immuno-Bed kit. Five-micrometer sections were cut using a Leica 2055 microtome and counterstained with eosin. Sections were analyzed using a Zeiss Axiovert100 microscope and pictures were taken using an Olympus Magnafire color camera.

Note: Per convention, we refer to human genes in using all upper case letters (*CYP26A1*), murine genes with only the first letter in upper case (*Cyp26a1*), and zebrafish gene in small case letters (*cyp26a1*).

Results

***Cyp26a1* is induced in Apc^{MIN} mouse adenomas and human FAP adenomas and human sporadic carcinomas.** Mutations in *APC* are implicated in the progression of colon cancer. Our previous findings that human colon tumors are deficient in retinoic acid biosynthesis prompted us to examine whether *CYP26A1*, a major retinoic acid catabolic enzyme, was also up-regulated in mice and humans with *APC* mutations. To evaluate this, both wild-type littermates and Apc^{MIN} heterozygotes were grown to 12 to 16 weeks and sacrificed. Adenomas and normal tissue of the colon were dissected out and total RNA was isolated. Quantitative reverse transcription-PCR (RT-PCR) indicated that *Cyp26a1* was significantly elevated in murine colon adenomas compared with normal tissues (Fig. 1*A*). We then looked at *CYP26A1* expression in human FAP colon adenomas. As seen in mice, RT-PCR analysis for *CYP26A1* expression from patient-matched uninvolved and FAP adenomas showed that all samples tested had elevated *CYP26A1* expression (Fig. 1*B*). Extending these findings, we also found that sporadic human colon tumor samples expressed significantly higher levels of *CYP26A1* compared with normal tissues (Fig. 1*C*). Up-regulation of *CYP26A1* in both mice and humans with *APC* mutations supports the idea that dysregulation of CYP26A1 plays a role in the development of colorectal carcinoma cancer.

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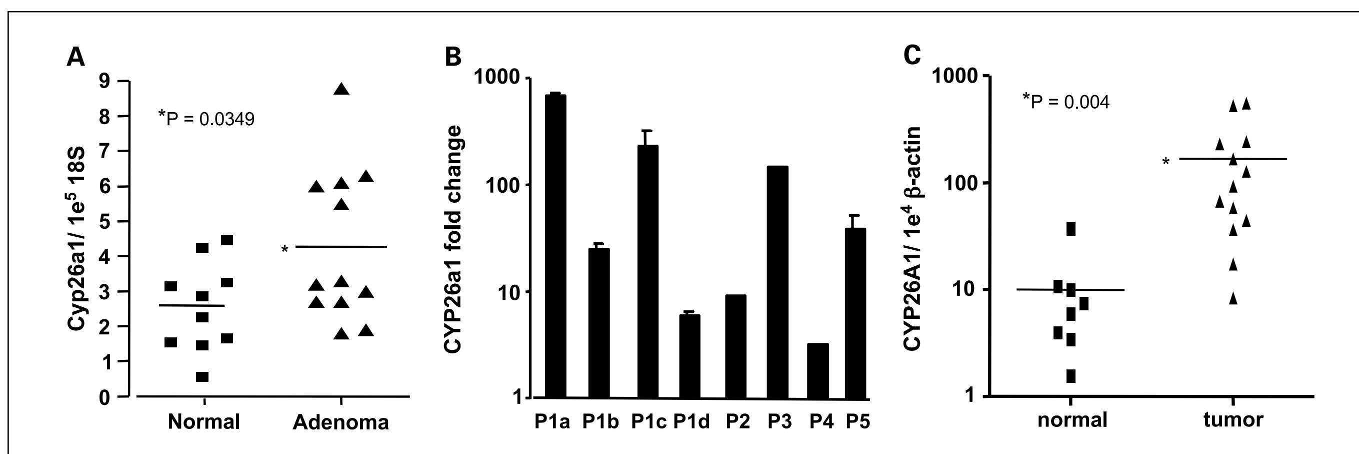

Figure 1. *CYP26A1* is high in murine and human adenomas and human sporadic carcinoma samples. *A,* quantitative RT-PCR of *Cyp26a1* expression in Apc^{MIN} mouse colon adenomas. Levels of Cyp26a1 were normalized in comparison to levels of 18S ribosomal subunit RNA. *B, CYP26A1* expression in matched human FAP adenomas. Values are fold-change in adenomas relative to patient-matched uninvolved tissue. P1-5 refer to individual patients, while P1a-d refer to separate samples from one patient. Error bars refer to *s*tandard *d*eviations (SD) of three measurements. *C, CYP26A1* expression in human sporadic carcinoma samples normalized to β-actin.

***Cyp26a1* is up-regulated in apc-deficient zebrafish.** The above findings in murine and human adenomas suggested dysregulation of CYP26A1 as an early event following *APC* mutation. Recent studies have shown that homozygous apc^{mcr} mutant and *apc* antisense knockdown zebrafish embryos harbor a number of developmental defects consistent with loss of retinoic acid, including pancreatic and intestinal differentiation defects (20, 23). In addition, adult zebrafish carrying heterozygous *apc* mutations develop spontaneous adenomas in the liver, pancreas, and intestine (26). We, therefore, examined the relationship between *apc* and *cyp26a1 in vivo* using apc^{mcr} mutant and morphant zebrafish. Whole-mount *in situ* hybridization showed that *cyp26a1* is expressed at very low levels in the brachial arches and neural retina at 80 hpf of wild-type embryos. In contrast, however, *cyp26a1* expression is strongly up-regulated in the brachial arches, neural retina, and gut of apc^{mcr} mutant and morphant embryos. Quantitative RT-PCR analysis revealed a 3- to 4-fold increase in *cyp26a1* expression in either apc^{mcr} mutant or *apc* morphant embryos compared with wild-type embryos (Fig. 2*A* and *B*). Histologic cross-sectioning showed that the gut-specific expression of *cyp26a1* was confined to the epithelium throughout the intestinal tube (Fig. 2*C*). To further examine this divergence in expression from wild type, we analyzed expression levels in wild-type and apc^{mcr} mutants over a developmental time course starting at 48 hpf when the gross morphology of homozygous apc^{mcr} mutant embryos could be confidently distinguished from wild-type siblings. Expression of *cyp26a1* was 1.8-fold higher in apc^{mcr} mutants at 48 hpf and gradually increased to 3- to 4-fold at ~80 hpf when apc^{mcr} mutant embryos begin to die. This increase in expression was due to increasing *cyp26a1* mRNA levels in mutants relative to *β-actin*, as well as decreasing expression of *cyp26a1* in wild-type embryos (Fig. 2*D*).

***Cyp26a1* expression in apc^{mcr} mutants is not due to retinoic acid induction.** Previous studies have shown that *cyp26a1* is strongly regulated by retinoic acid, its chemical substrate (12). Indeed, *cyp26a1* can be induced in the gut of 80 hpf zebrafish by

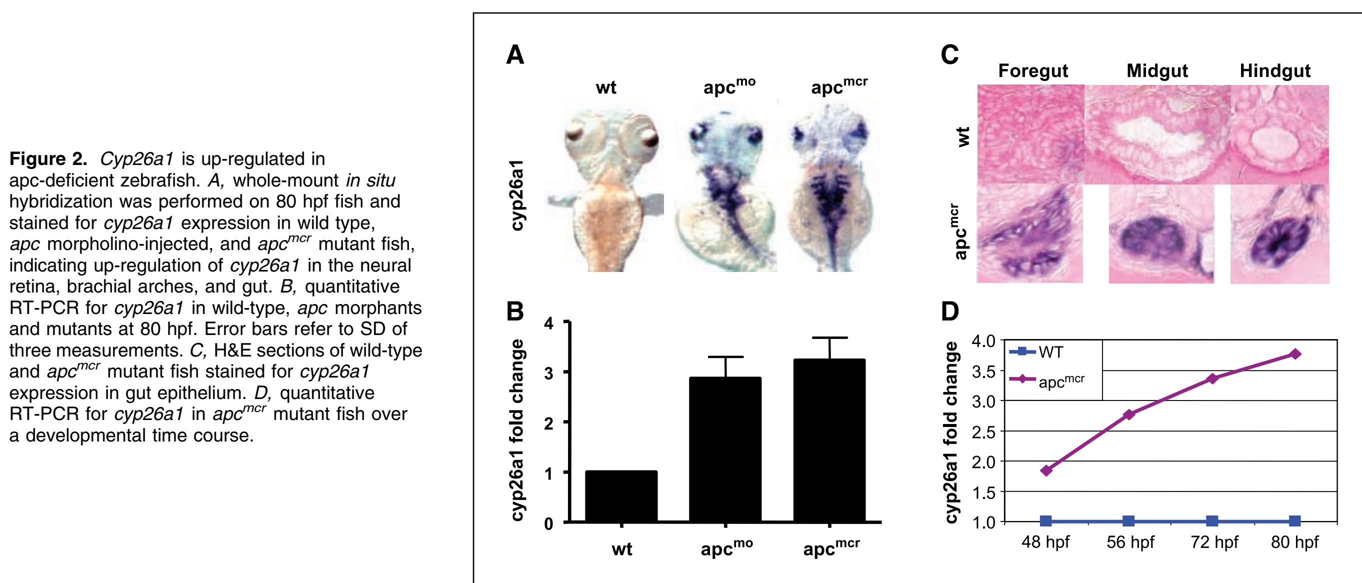

Figure 2. *Cyp26a1* is up-regulated in apc-deficient zebrafish. *A,* whole-mount *in situ* hybridization was performed on 80 hpf fish and stained for *cyp26a1* expression in wild type, *apc* morpholino-injected, and apc^{mcr} mutant fish, indicating up-regulation of *cyp26a1* in the neural retina, brachial arches, and gut. *B,* quantitative RT-PCR for *cyp26a1* in wild-type, *apc* morphants and mutants at 80 hpf. Error bars refer to SD of three measurements. *C,* H&E sections of wild-type and apc^{mcr} mutant fish stained for *cyp26a1* expression in gut epithelium. *D,* quantitative RT-PCR for *cyp26a1* in apc^{mcr} mutant fish over a developmental time course.

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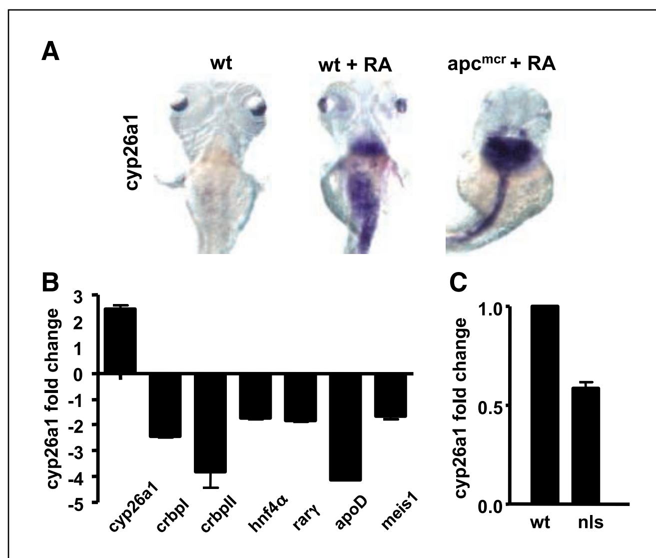

Figure 3. RA induces *cyp26a1* expression but is not responsible for its induction in *apc^{mcr}* mutants. *A,* whole mount *in situ* hybridization of 80 hpf embryos stained for *cyp26a1* in wildtype, RA-treated wild-type and *apc^{mcr}* mutant fish. *B,* quantitative RT-PCR of known RA-target genes in *apc^{mcr}* mutants. *C,* quantitative RT-PCR of *cyp26a1* in *raldh2* mutant fish, *neckless* (*nls*). Error bars refer to SD of three measurements.

the addition of retinoic acid (Fig. 3*A*). However, previous studies have also shown that *apc^{mcr}* mutants are retinoic acid deficient (20, 23). To verify that *apc^{mcr}* mutants are retinoic acid deficient, we did RT-PCR on 80 hpf wild type and *apc^{mcr}* mutant fish and examined the expression of known retinoic acid target genes. In this analysis, *crbp1, crbp2, hnf4α, rarγ, apoD*, and *meis1* were all down-regulated by 2-fold compared with wild-type embryos (Fig. 3*B*). This indicates that *cyp26a1* up-regulation was not due to the presence of retinoic acid. In addition, treatment of *apc^{mcr}* mutant embryos with 10 μmol/L 4-(*N,N*-diethylamino) benzaldehyde (DEAB) to inhibit retinoic acid production failed to reduce levels of *cyp26a1* in *apc^{mcr}* mutants, compared with wild type (data not shown). Finally, *raldh2* mutant embryos (*nls*), which are also retinoic acid deficient and share a number of phenotypes with *apc^{mcr}* mutant embryos, did not exhibit increased levels of *cyp26a1* (Fig. 3*C*), indicating that up-regulation of *cyp26a1* in *apc^{mcr}* mutant embryos is not simply due to retinoic acid deficiency (7). These observations indicated that *cyp26a1* elevation in *apc^{mcr}* mutants was specifically due to loss of *apc*.

***Cyp26a1* is a WNT/β-catenin/TCF target gene.** Because the targets of the WNT/β-catenin/TCF pathway are up-regulated in the presence of APC mutations, we examined the response of *cyp26a1* expression to perturbation of several components in this pathway. First, we designed a splice-blocking antisense oligonucleotide morpholino directed against *tcf4*, the TCF expressed in the gut epithelium (27). We injected this into an *apc^{mcr}* mutant carrying an integrated TOPdGFP transgene (28) to verify interrupted WNT signaling. Full knockdown of *tcf4* showed severe lethality (data not shown). As such, we injected sublethal doses of the antisense into one- to two-cell embryos and harvested embryos for whole-mount *in situ* and quantitative RT-PCR at 56 and 80 hpf. Expression of *cyp26a1* decreased in the brachial arches, eyes, and gut of *tcf4* antisense-injected mutant embryos (Fig. 4*A-C*). In addition, injection of dominant negative LEF (dnLEF), a form of LEF unable to bind β-catenin, reduced cyp26a1 expression. In both cases, the reduction in *cyp26a1* was paralleled by decreased expression of the control green fluorescent protein (GFP) reporter construct (Fig. 4*D*; ref. 28).

We next looked at overexpressing β-catenin by injecting mRNA of the stabilized form of β-catenin, S37A, into wild-type embryos at the one-cell stage at 0, 5, 10, 15, and 25 pg amounts. Expression of *cyp26a1* rose in a dose-dependent manner, along with another

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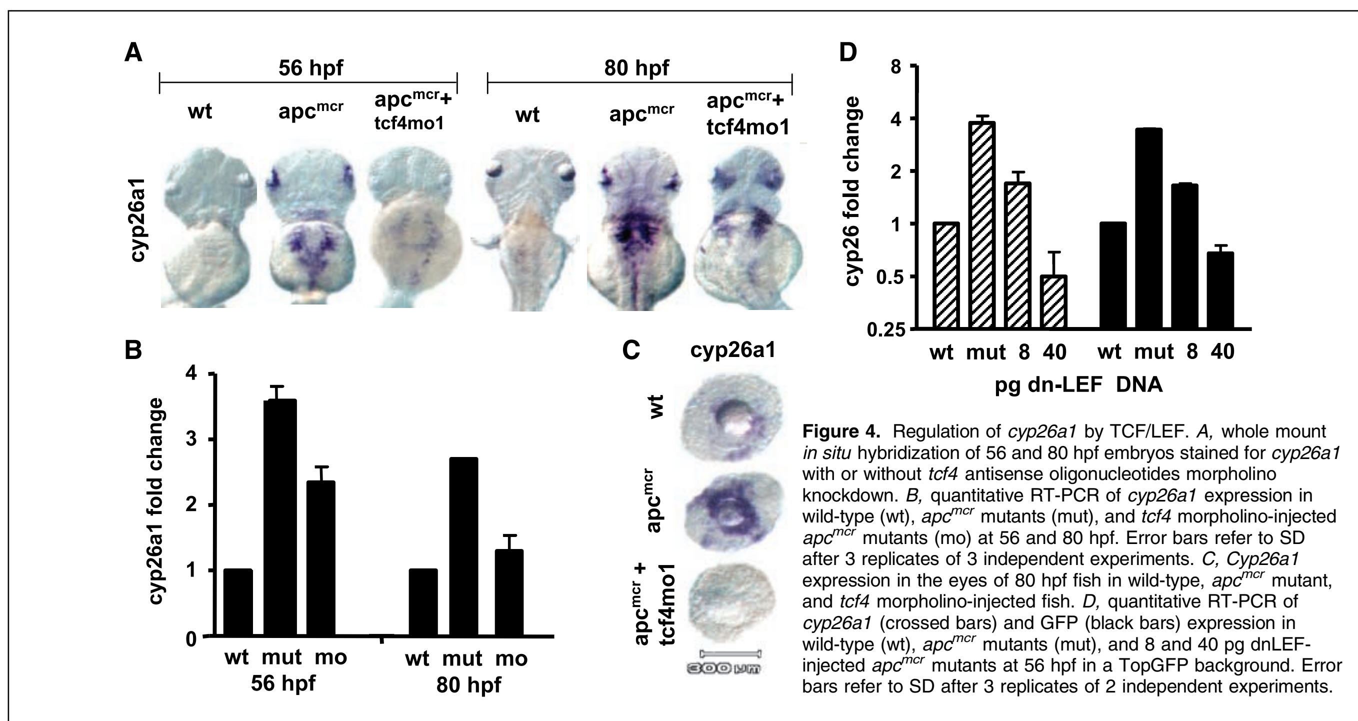

Figure 4. Regulation of *cyp26a1* by TCF/LEF. *A,* whole mount *in situ* hybridization of 56 and 80 hpf embryos stained for *cyp26a1* with or without *tcf4* antisense oligonucleotides morpholino knockdown. *B,* quantitative RT-PCR of *cyp26a1* expression in wild-type (wt), *apc^{mcr}* mutants (mut), and *tcf4* morpholino-injected *apc^{mcr}* mutants (mo) at 56 and 80 hpf. Error bars refer to SD after 3 replicates of 3 independent experiments. *C, Cyp26a1* expression in the eyes of 80 hpf fish in wild-type, *apc^{mcr}* mutant, and *tcf4* morpholino-injected fish. *D,* quantitative RT-PCR of *cyp26a1* (crossed bars) and GFP (black bars) expression in wild-type (wt), *apc^{mcr}* mutants (mut), and 8 and 40 pg dnLEF-injected *apc^{mcr}* mutants at 56 hpf in a TopGFP background. Error bars refer to SD after 3 replicates of 2 independent experiments.

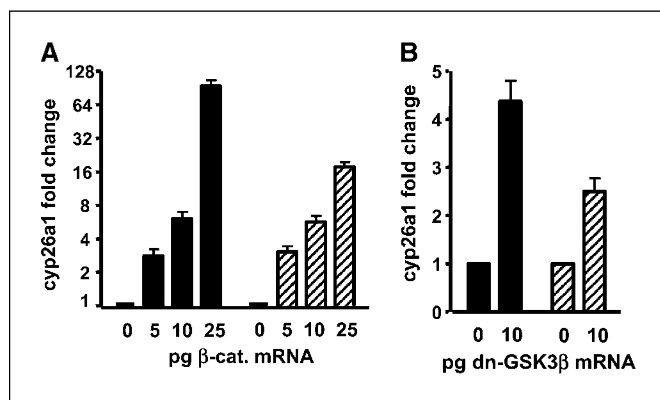

Figure 5. Regulation of *cyp26a1* by β-catenin. *A,* dose-dependent up-regulation of *mmp13* (*black bars*) and *cyp26a1* (*crossed bars*) at 80 hpf following injection of *β-catenin* mRNA into wild-type embryos. *B,* induction of *mmp13* (*black bars*) and *cyp26a1* (*crossed bars*) at 80 hpf following injection of 10 pg *dnGSK3β* mRNA into wild-type embryos. Error bars refer to SD of 3 replicates in 3 independent experiments.

WNT target gene, *matrix metalloproteinase 13* (*mmp13*; Fig. 5*A*; ref. 29). Further, injection of 10 pg of a dominant-negative form of *GSK3β* mRNA, which stabilizes β-catenin levels, also caused *cyp26a1* and *mmp13* expression to increase, 2- and 4-fold, respectively (Fig. 5*B*). These data indicate that cyp26a1 is a target of the WNT/β-catenin/TCF pathway.

Inhibition of *cyp26a1* increases intestinal differentiation. We have shown previously that apc^{mcr} mutants lack markers of terminal differentiation in the gut, pancreas, eye, and liver and that addition of retinoic acid rescued these terminal differentiation markers (20, 23). Because cyp26a1 is largely responsible for degrading retinoic acid (30), we hypothesized that *cyp26a1* overexpression contributed to the lack of terminal differentiation seen in the intestines of apc^{mcr} embryos. To test this idea, we designed an antisense morpholino to block *cyp26a1* splicing and injected it into apc^{mcr} mutant embryos along with parallel injections of control oligonucleotides. Confirmation of *cyp26a1* splice blocking was provided by quantitative RT-PCR for the unspliced product (data not shown). Following confirmation of *cyp26a1* knockdown, we examined control and *cyp26a1* morphant embryos for expression of the intestinal differentiation marker *i-fabp* by whole-mount *in situ* hybridization. A parallel set of embryos was treated with retinoic acid as previously described and served as a positive control. As expected, treatment of embryos with retinoic acid increased *i-fabp* expression with ~30% (n = 92) of the embryos staining positive. Similarly, knockdown of *cyp26a1* increased the number of *i-fabp*-positive guts in apc^{mcr} mutants compared with ~60% (n = 57). This was in contrast to control injected embryos with only 3% positive staining for *i-fabp* (Fig. 6*A* and *B*).

To further assess the role of cyp26a1 in preventing differentiation in apc^{mcr} mutants, we synthesized a known small-molecule inhibitor of cyp26a1, a RAMBA (25). We treated apc^{mcr} mutants with vehicle or RAMBA at (900 nmol/L) and determined the number of embryos that stained positively for the expression of *i-fabp*. Apc^{mcr} mutants treated continuously with RAMBA for 4 days starting at 70% epiboly exhibited 70% (n = 35) rescue of terminal marker *i-fabp* compared with the ~30% rescue with retinoic acid alone (Fig. 6*A* and *B*). In addition to the standard treatment, treatment with RAMBA starting at 48 and 72 hpf led to some rescue, ~70% (n = 25) and ~30% (n = 36), respectively, whereas treatment with retinoic acid starting at 48 or 72 hpf was without effect. Therefore, inhibition of retinoic acid catabolism by interference with cyp26a1 activity, either by antisense knockdown or pharmacologic inhibition, leads to increased terminal differentiation in apc^{mcr} mutant fish.

Discussion

Impaired retinoic acid signaling has been found in a large number of cancers, including those of the head, neck, colon, breast, prostate, and kidney (14, 30–32). This dysregulation can occur at multiple levels, including loss of retinoic acid receptor function, vitamin A absorption and storage defects, impaired retinoic acid biosynthesis, and enhanced retinoic acid catabolism (20, 23, 31–34). Although deficiencies have been documented for each of these steps, we know little about the mechanisms underlying these alterations or whether they are causative in tumor formation. Here, we show that increased expression of *CYP26A1*, the major retinoic acid–metabolizing cytochrome P450 enzyme in the intestine (35), occurs as a direct consequence of mutations in the colon tumor suppressor gene, *APC*. In addition, we show for the first time that *CYP26A1* is regulated by the WNT signaling pathway and that overexpression of *CYP26A1* within the intestine impairs intestinal differentiation. Our data suggest a model wherein APC plays a role

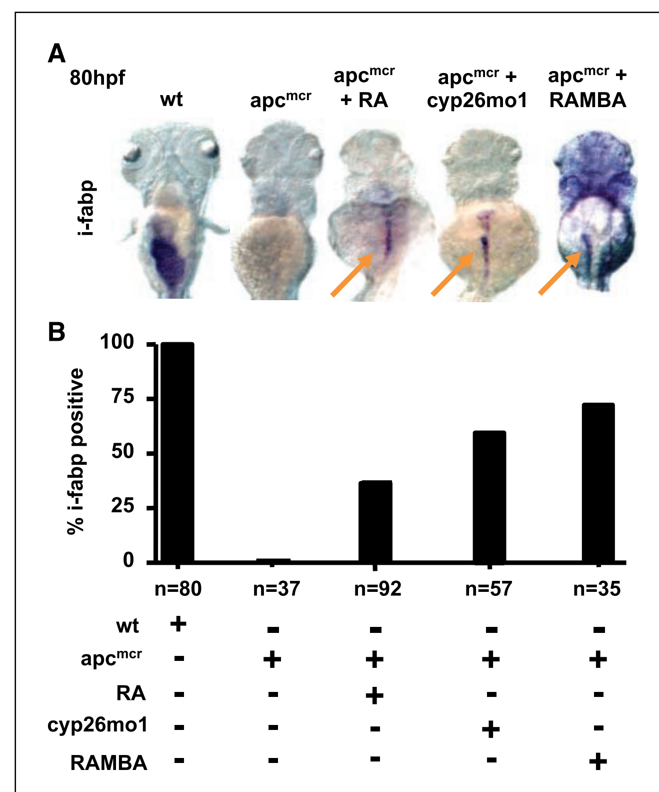

Figure 6. Reduction of cyp26a1-mediated RA catabolism leads to increased terminal differentiation. *A,* whole-mount *in situ* hybridization of 80 hpf embryos stained for gut terminal differentiation marker *i-fabp* (arrows) in wild-type, apc^{mcr} mutants, apc^{mcr} mutants treated with retinoic acid, apc^{mcr} mutants injected with splice-blocking morpholino to *cyp26a1*, and apc^{mcr} mutants treated with RAMBA. *B,* embryos scored for presence of *i-fabp* in the gut in wild-type, apc^{mcr} mutants, apc^{mcr} mutants treated with retinoic acid, apc^{mcr} mutants injected with splice-blocking morpholino targeted to *cyp26a1*, and apc^{mcr} mutants treated with RAMBA.

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in promoting intestinal cell differentiation by suppressing the catabolism of retinoic acid.

A number of recent studies indicate that the up-regulation of *CYP26A1* might contribute to the formation of carcinomas. For example, high levels of *CYP26A1* were reported in head and neck cancers (32). Similarly, breast and colon carcinoma cell lines seem to harbor high levels of *CYP26A1* (30). Further, retinoic acid resistance in acute promyelocytic leukemia patients has been attributed to increased CYP26A1 activity (13, 14). Finally, a recent finding by Osanai et al. (36) indicates that expression of *Cyp26a1* protects cells from apoptosis, a key hallmark of malignant cells. The finding that *CYP26A1* levels are increased in *APC* mutant tissues of zebrafish, mice, and humans agrees with previous reports implying a role for CYP26A1 in carcinoma formation. However, our studies extend these findings in a number of ways. First, the up-regulation of *CYP26A1* is dependent on loss of *APC*, a major colon tumor suppressor gene. Moreover, the induction of *cyp26a1* seems to precede carcinoma formation in that the intestines of apc^{mcr} mutant zebrafish embryos at 80 hpf already show robust up-regulation. Similarly, APC-dependent adenomas from mice and humans also showed *CYP26A1* induction. Interestingly, *cyp26a1* induction in homozygous apc^{mcr} mutant zebrafish embryos occurred in the absence of any evident formation of intestinal neoplasms, thereby suggesting that up-regulation of *cyp26a1* is part of the initiating events following apc loss. Consistent with *CYP26A1* dysregulation as an early event, we found that its induction is dependent on WNT signaling, a well described consequence of APC loss.

The control of CYP26A1 by APC and WNT provides evidence for a dual role for APC in the control of both retinoic acid production as well as retinoic acid catabolism. Recent studies indicate that loss of APC results in loss of expression of retinoic acid biosynthetic enzymes like *DHRS9* in humans and *rdh1* and *rdh1l* in the zebrafish intestine (19, 20, 23). Combined with our current findings, it seems that APC may switch intestinal cells between a retinoic acid synthetic state and a retinoic acid catabolic state, thereby helping to promote intestinal cell differentiation. This mechanism would allow for the precise control of retinoic acid levels within cells at various stages of maturation and differentiation within the intestine. For example, stem cells undergoing self-renewal in response to WNT stimulation could be protected from retinoic acid effects by up-regulation of *CYP26A1*, a notion supported by the fact that *Cyp26a1* was originally identified in mouse embryonic stem cells (37). Indeed, this type of juxtaposition of retinoic acid production with retinoic acid catabolism occurs in a number of developmental settings. Koubova et al. (38) recently found that the expression of *Cyp26b1* determines expression of *Stra8*, which determines sex-specific regulation of germline meiosis in stem cells. Further, *Cyp26b1* has also been shown to protect the distally expanding developing mouse limb bud from retinoic acid–mediated differentiation initiated at the proximal trunk, coordinately regulating proliferation with differentiation (9). Finally, the dorsal/ventral patterning of the developing chick retina is determined by the expression of several Cyp26 isoforms and retinoic acid (39).

The regulation of *CYP26A1* by WNT is significant because previous studies of *CYP26A1* expression have defined retinoic acid as its only known direct regulator. Indeed *CYP26A1* is a well-known, direct target of ATRA (12). It seems, however, that the up-regulation of *CYP26A1* following APC loss is independent of retinoic acid. Several lines of evidence support this possibility. First, apc^{mcr} mutant zebrafish lack RDHs and show profound retinoic acid deficiency phenotypes such as lack of pectoral fins and lack of jaw development (20, 23). In agreement with this finding, we found that a number of retinoic acid target genes, including *crbpI, crbpII, hnf4α, rarγ, apoD*, and *meis1* were down-regulated in apc^{mcr} mutants, whereas *cyp26a1* was up-regulated (40–44). In addition, treatment of apc^{mcr} mutant embryos with DEAB, a raldh2 inhibitor failed to suppress *cyp26a1* levels. As such, it seems the induction of *cyp26a1* in the apc^{mcr} mutant zebrafish is not a result of increased retinoic acid biosynthesis. Regulation of *cyp26a1* through retinoic acid–independent pathways is in agreement with findings by Dobbs-McAuliffe et al. (45) showing the continued expression of *cyp26a1* in the presence of DEAB in the caudal end of the developing zebrafish embryo, as well as, by Iulianella et al. (46) in mouse embryos.

A number of previous studies in rodents support the use of retinoic acid in preventing colon carcinogenesis. For example, several retinoids suppressed aberrant crypt formation in F344 rats treated with azoxymethane (47). Similarly, ATRA reduced the number of aberrant crypts in Sprague-Dawley rats exposed to the same carcinogen (48). In contrast, however, a recent study using ATRA in the Apc^{MIN} mouse found enhanced adenoma formation within the small intestine. However, no changes in adenoma formation were observed within colon of Apc^{MIN} mice following treatment with ATRA (22). It is unclear what accounts for the difference in response between adenomas of the small intestine and the colon to ATRA. Our studies examined CYP26A1 expression in adenomas taken from the colon of Apc^{MIN} mice, as well as human FAP adenomas and sporadic carcinomas taken from the colon. It is possible that the small intestine and colon respond differently to retinoic acid and that up-regulation of *Cyp26a1* in colon adenomas of Apc^{MIN} mice may have protected colon adenomas from the action of ATRA. The complexities of retinoic acid responses in the intestine require further investigation to resolve these apparent paradoxical findings.

We found that genetic or pharmacologic inhibition of *cyp26a1* alone reversed intestinal differentiation defects in apc^{mcr} mutant zebrafish. Our findings are in agreement with previous studies supporting the notion that CYP26A1 represents a potential pharmacologic target for the treatment of cancer. For example, low-dose treatment of MCF7 cells with liarozole, a specific CYP26 inhibitor, and retinoic acid led to significant increases in differentiation, reduction of proliferation, cell adhesion, and metastatic potential (49). Similarly, R116010, another highly specific CYP26 inhibitor, inhibited the growth of mammary tumor cells (50). Finally, Njar et al. (23, 25) recently found that treatment with novel synthetic RAMBAs resulted in increased apoptosis, differentiation, and a reduction in tumor size in mouse xenograft models. Taken together, the above findings indicate a need for continued investigation into the possibility of improving retinoic acid responsiveness through simultaneous treatment with retinoic acid and CYP26A1 inhibitors.

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