Adenomatous polyposis coli control of retinoic acid biosynthesis is critical for zebrafish intestinal development and differentiation.

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Abstract

Mutations in the adenomatous polyposis coli (APC) tumor suppressor gene cause uncontrolled proliferation and impaired differentiation of intestinal epithelial cells. Recent studies indicate that human colon adenomas and carcinomas lack retinol dehydrogenases (RDH) and that APC regulates the expression of human RDHL. These data suggest a model wherein APC controls enterocyte differentiation by controlling retinoic acid production. However, the importance of APC and retinoic acid in mediating control of normal enterocyte development and differentiation remains unclear. To examine the relationship between APC and retinoic acid biosynthesis in normal enterocytes, we have identified two novel zebrafish retinol dehydrogenases, termed zRDHA and zRDHB, that show strong expression within the gut of developing zebrafish embryos. Morpholino knockdown of either APC or zRDHB in zebrafish embryos resulted in defects in structures known to require retinoic acid. These defects included cardiac abnormalities, pericardial edema, failed jaw and pectoral fin development and the absence of differentiated endocrine and exocrine pancreas. In addition, APC or zRDHB morphant fish developed intestines that lacked columnar epithelial cells and failed to express the differentiation marker intestinal fatty acid binding protein (i-FABP). Treatment of either APC or zRDHB morphant embryos with retinoic acid rescued the defective phenotypes. Downstream of retinoic acid production, we identified hoxc8 as a retinoic acid induced gene that, when ectopically expressed, rescued phenotypes of APC and zRDHB deficient zebrafish. Our data establish a genetic link supporting a critical role for retinoic acid downstream of APC and confirm the importance of retinoic acid in enterocyte differentiation.
Introduction

Mutations in the tumor suppressor gene, *adenomatosus polyposis coli (APC)*, result in an inherited colon cancer predisposition known as familial adenomatous polyposis (FAP) (1-3). In addition, recent studies on sporadic, non-polyposis neoplasms indicate that approximately 85% of colon carcinomas carry mutations within the *APC* gene (4). Given the importance of APC loss in colorectal cancer, intense investigation has focused on defining the molecular mechanisms controlled by APC. These efforts have resulted in a model wherein APC plays a critical role in controlling colonocyte proliferation by regulating the WNT signaling pathway (1-3). In this model, APC serves as a cytoplasmic scaffolding molecule that permits assembly of a complex containing axin, glycogen synthase kinase-3β and casein kinase Iε, which work together to target the transcriptional co-activator, β-catenin, for ubiquitin-mediated degradation (1-3,5,6). APC-dependent destruction of β-catenin prevents it from associating with TCF-LEF transcription factors in the nucleus, thereby blocking activation of pro-proliferative target genes such as *c-myc* (7) and *cyclin D1* (8). In cells lacking functional APC, β-catenin accumulates and translocates into the nucleus where it serves as a co-activator for TCF-LEF transcription factors in inducing a program of cell proliferation. Support for β-catenin as a downstream target of APC comes from studies demonstrating that mutations in β-catenin account for an additional 7% of sporadic colon carcinomas (6). These mutations render β-catenin resistant to ubiquitin-mediated proteolysis and may substitute for loss of *APC* in these carcinomas (6). It is unclear, however, whether the tumor suppressor functions of APC are limited to its regulation of β-catenin and whether loss of β-catenin control following *APC* mutation accounts fully for the clinical phenotypes following *APC* mutation (9).

Recent studies indicate that APC may promote colonocyte differentiation by stimulating the production of retinoic acid (RA), a biologically active lipid mediator with important roles controlling cell fate and differentiation (10). Central to the ability of a cell to respond to retinoic acid is the requirement of first converting dietary retinol (vitamin A) into retinoic acid, a process that occurs via two enzymatic
steps (11). The first step of this process converts retinol to retinal and is mediated by alcohol dehydrogenases (ADH) and short chain dehydrogenases (SDR). The second step involves conversion of retinal into retinoic acid via aldehyde dehydrogenases (ALDH) (11). Given this required biosynthetic conversion, retinoic acid production is limited to cells harboring the necessary enzymes for conversion of vitamin A.

We demonstrated previously that while colon adenomas and carcinomas have elevated β-catenin target genes, they also showed a deficiency of retinoic acid biosynthetic enzymes (10). In establishing a link between APC and control of retinoic acid biosynthesis, introduction of APC into an APC-mutant colon carcinoma cell line increased retinoic acid biosynthesis in parallel with the transcriptional induction of retinol dehydrogenase L (RDHL) (10). Despite these observations, we currently lack evidence confirming a pivotal role for APC and retinoic acid in controlling enterocyte development and differentiation in vivo. We have utilized zebrafish to examine the relationship between APC, retinoic acid biosynthesis and gut development. The data presented herein show that APC controls a retinoid and hox-dependent program of development and enterocyte differentiation in zebrafish. Key supporting evidence for this model comes from studies showing that morpholino knockdown of APC or the novel zebrafish retinol dehydrogenase, zRDHB, resulted in comparable defects in development including gut cell morphology and differentiation status. The phenotypes evoked by knockdown of either APC or zRDHB were rescued by addition of exogenous retinoic acid or injection of hoxC8 mRNA.
Materials and Methods

**Embryo culture and zebrafish stocks.** *Danio rerio* (zebrafish) were maintained on a 14 hour light:10 hour dark cycle and maintained at 28.5°C. Fertilized embryos were collected following natural spawnings, cultured and staged by developmental time and morphological criteria as described previously(12).

**Expression Analyses.** Adult zebrafish tissues were dissected and then homogenized in Trizol reagent (Invitrogen) using a Fastprep (Thermosavant) tissue homogenizer. Total RNA was then isolated according to manufacturer’s instructions.

Singled stranded cDNA was synthesized from 1 µg total RNA using Superscript III (Invitrogen). PCR primers used were as follows: zRDHA (tissue distribution): forward, 5’-CTCTTTGGAGGAGCTTACTGCAT-3’; reverse, 5’-AATATCGTGTCAGGATGAAC-3’, zRDHA (developmental time course): 5’-CTCTTTGGAGGAGCTTACTGCAT-3’; reverse, 5’-AATATCGTGTCAGGATGAAC-3’, zRDHB (tissue distribution): forward, 5’-ACTGAAAACGCTCCAGCTCAAT-3’; reverse, 5’-ACACCAGTCAGATTCACATCCA-3’, zRDHB (developmental time course): forward, 5’-TGATTGAAGACGACCTGAAGAA-3’; reverse, 5’-ACCAGGGCATTAGTGAAGATGT-3’, cdx1 forward, 5’-TTGGAGAAAGAGGCAAGCAT-3’, reverse, 5’-TCGGATTTTCTTCTGATTGTGA-3’, hoxc8: forward, 5’-AAGCGCCGTATTGAAGTGTCCC-3’; reverse, 5’-TCAGCTCCTTGTCCCTCCTTTCTCTC-3’, β-actin: 5’-GCTGCTGTGGATGCTGTGAT-3’; reverse, 5’-AGAGTCCATCAGCATAACAG-3’. A template-free negative control was included in each experiment.

Quantitative RT-PCR was performed using the Roche Light Cycler instrument and software, version 3.5 (Roche Diagnostics). Primers used were as follows: cyclin D1: forward, 5’-ggaactgctggcgctaaata-3’; reverse, 5’-gacttgcgagaggaagttgg-3’, c-myc: forward, 5’-tgactgtggaaaagcgacag-3’; reverse, 5’-gctgctgttgatgctgtgat-3’, hoxc8a: forward, 5’-aagcgccgttattgaagtgtccc-3’; reverse, 5’-tcactccttctcttctctcttc-3’. PCR reactions were performed in
duplicate using the LightCycler FastStart DNA Master SYBR Green I kit (Roche). PCR conditions were as follows: 35 cycles of amplification with 10 seconds denaturation at 95°C, and 5 second annealing at 57°C. A template-free negative control was included in each experiment.

**Whole-mount in situ hybridizations.** For whole-mount *in situ* hybridizations, embryos were fixed in 4% paraformaldehyde in sucrose buffer, rinsed in PBS, dehydrated in methanol and stored at -20°C. *In situ* hybridizations were carried out as described. Digoxigenin-labeled riboprobes were generated by linearization of pCRII (Invitrogen) containing zRDHA, zRDHB, i-FABP, insulin, trypsin, or *hoxc8* cDNA followed by in vitro transcription with SP6 or T7 RNA polymerase (Roche). Embryos were cleared in 70% glycerol/PBS and photographed with a Leica MZ12 dissecting microscope(13).

**Morpholino and RNA microinjections.** Morpholino oligos were obtained form Gene Tools LLC. The RDHB splicing blocking morpholino (5’- atccagtggcactcacctttcccg-3’), APC morpholino (5’-tagcatactctacctgtgctcttcg-3’) and control morpholino (5’-cctcttacctcagttacaatttata-3’) were solubilized to 1 mM in 1x Danieau buffer. For microinjections, 0.5 mM morpholino was injected into wildtype embryos at the one to four cell stages(14).

For *hoxc8* rescue experiments, full-length Hoxc8 RNA was transcribed from a linearized pCRII/hoxc8 construct using mMessage mMachine (Roche) according to the manufacturer’s protocol. For injections, 150 pg of capped and polyadenylated *hoxc8* mRNA or 150 pg of capped and polyadenylated GFP mRNA was co-injected with an RDHB MO or a standard control morpholino into one-two cell stage embryos.

**Retinoic Acid Treatments.** To rescue zRDHB morphants by application of retinoic acid, embryos were incubated in 1µM all-trans retinoic acid in DMSO at 50% epiboly for one hour. Embryos were then washed in embryo water. RA treatments were repeated every 24 hours for one hour. Control embryos were treated over these periods with an equal volume of DMSO(15).
**RA Extraction and HPLC Analysis.** Cells were treated with 100 nmol ATROL at 80-90% confluence for 8 hours. Media was removed and cells were scraped into PBS for protein or RNA quantification. After addition of 100 nmol internal standard TTNPB, the media was acidified with 6N HCl (0.03X volume) and extracted with equal volume of hexane containing 0.1 mg/mL butylated hydroxytoluene. The organic phase was transferred to a glass vial, dried under nitrogen and reconstituted in 100 µl 1:1 DMSO/MeOH for HPLC analysis. Retinoid quantities were determined as previously described ((16)).

**Histological Analyses.** Embryos were fixed in 4% paraformaldehyde in sucrose buffer, rinsed in PBS and embedded in paraffin. Six-micron sections were cut using a Leica microtome and stained in hematoxylin and eosin. Sections were analyzed using an Olympus compound microscope and pictures were taken using a Zeiss Axiocam.
Results

*Phenotypes of APC knockdown zebrafish are rescued by retinoic acid*

To examine the hypothesis that APC facilitates enterocyte differentiation by regulating retinoic acid biosynthesis, we sought to knock down APC function and determine whether any resulting phenotypes were comparable to knockdown of retinoic acid biosynthetic enzymes. To accomplish this, we designed an APC-specific splicing blocking morpholino that targeted the splicing of APC between exon 15 and intron 15. Injection of this APC morpholino into wildtype zebrafish embryos at the one-to-two cell stage resulted in the production of an intron-retained RNA transcript as verified by rt-PCR (Fig. 1A). This corresponded with a reduction in the level of the fully spliced APC transcripts. Analysis of mRNAs from embryos injected with a control morpholino demonstrated only fully spliced APC transcripts (Fig. 1A). To further validate APC knockdown, we measured the expression levels of *c-myc* (7) and *cyclin D1* (8), which are reported β-catenin target genes and, therefore, should be up-regulated following APC knockdown. Quantitative rt-PCR analysis confirmed increased expression of these β-catenin target genes in the APC deficient embryos (Fig. 1B).

Having verified APC knockdown, we analyzed the resulting morphant embryos and observed a number of defects consistent with loss of retinoic acid including pericardial edema, failed cardiac looping, loss of jaw formation and arrested pectoral fins (Fig. 1C) (15,17,18). Furthermore, the morphant embryos showed reduced staining for the differentiated pancreas markers, insulin and trypsin, both known to require retinoic acid (Fig. 1D) (17). All of these phenotypes were strikingly similar to those reported for the zebrafish *neckless* mutant which carries a mutation in the retinaldehyde dehydrogenase-2 gene and is defective in retinoic acid biosynthesis (15). In addition, the cardiac, jaw and fin phenotypes present in the APC morphants are identical to those seen in a recently described APC mutant zebrafish (19).

We attempted to rescue APC morphant phenotypes by application of exogenous retinoic acid. We found that treatment of morphant embryos with 1.0 µM RA once a day for four days rescued pectoral fin and jaw development in 65% (n=117) of the APC morphant embryos (Fig. 1C). In addition,
retinoic acid treatment rescued expression of the pancreatic markers trypsin and insulin in a similar proportion of animals (Fig. 1D).

**zRDHA and zRDHB are Novel Zebrafish Retinol Dehydrogenases**

Since retinoic acid treatment rescued APC morphant embryos and since APC induces the expression of human RDHL, we sought to identify zebrafish retinol dehydrogenases that might be downstream of APC. To accomplish this, we first used the human RDHL protein sequence to perform a tBLASTn search of all available zebrafish EST sequences. This search identified two independent zebrafish genes that we termed, zRDHA and zRDHB. We confirmed the presence of the predicted mRNAs for zRDHA and zRDHB in zebrafish tissues by designing primers that flanked the putative open reading frames and used these to amplify the corresponding cDNAs by rt-PCR. Using a pool of zebrafish gut mRNA, we amplified products of the predicted size for both zRDHA and zRDHB and confirmed the database-derived sequence for each gene by standard sequence analysis (data not shown). The Genbank accession numbers of these corresponding cDNAs are NM_199609 and NM_198069 for zRDHA and zRDHB, respectively.

Inspection of the protein alignments indicated that zRDHA and zRDHB were 47.0% and 45.5% identical to hRDHL, respectively, and that each displayed important structural features characteristic of known short chain fatty acid dehydrogenases/reductases (Fig. 2) (20). These features included a conserved cofactor binding site motif of GXXXGXG starting at Gly-36 in zRDHA and Gly-46 in zRDHB. In addition, each predicted protein also displayed an active site consensus sequence of YXXXK. The active site consensus sequence started at position Y-176 in zRDHA and Y-186 in zRDHB. Phylogenetic alignment of the predicted zRDHA and zRDHB proteins also suggested evolutionary conservation with retinol dehydrogenases (Fig 3A).

To validate zRDHA and zRDHB as enzymatically active retinol dehydrogenases, we over-expressed each protein fused to an N-terminal V5-tag in the human colon cell line, HCT116. Western blotting against the V5-tag confirmed protein expression and allowed optimization of protein expression
prior to enzyme activity analysis (data not shown). To assess enzymatic activity, we incubated cells carrying empty vector, V5-zRDHA, or V5-zRDHB with 100 nmol retinol for 18 hours and extracted retinoids from the culture media using acidified hexane. Retinoid derivatives in the extracts were separated by RP-HPLC and retinoic acid levels quantified by comparison to the internal extraction standard, TTNPB. Figure 3B shows that cells transfected with zRDHA or zRDHB produced approximately 3.0-fold more retinoic acid than cells transfected with vector alone, thus confirming zRDHA and zRDHB as bonafide retinol dehydrogenases.

**Tissue Distribution of zRDHA and zRDHB**

Sequence similarities within the short chain fatty acid dehydrogenase/reductase family make it difficult to precisely determine inter-species orthology based solely on protein alignments. We were unable, therefore, to determine whether zRDHA or zRDHB were direct orthologs of human RDHL. Previous studies, however, have shown that human RDHL is highly expressed in adult colon tissues, thus suggesting a specific functional role for RDHL in human gut (10,21). We, therefore, asked whether zRDHA or zRDHB showed gut restricted expression in zebrafish. To accomplish this, we first examined whether expression of zRDHA and zRDHB paralleled embryologic development of the gut, which in zebrafish is histologically evident beginning at 21 hpf (22,23). Rt-PCR analysis of zRDHA and zRDHB using RNA harvested from zebrafish embryos at various times between 0 and 120 hpf demonstrated expression of zRDHA in embryos by six hpf and remained strong at all time points examined through 120 hpf (Fig. 4A). zRDHB levels were very low and undetectable at time zero. By six hours, however, the levels of zRDHB began to increase and reached maximal levels at approximately 48 hpf. Expression of zRDHB remained robust through 120 hpf (Fig. 4A).

Whole mount *in situ* hybridization analyses on embryos at 48 and 96 hpf showed specific embryonic structures expressing *zRDHA* and *zRDHB*. At 48 hpf, the expression patterns of *zRDHA* and *zRDHB* were very similar and evident in anterior portions of the fish including the brain, pectoral fins and spine (Fig. 4B). At 48 hours there was no apparent staining of zRDHA or zRDHB in the
developing gut. By 96 hpf, however, the expression of the two RDHs was largely confined to the gut. This expression in the gut was confirmed by comparison to the expression of i-FABP (Fig. 4B), a known marker of mature gut epithelium (24). As expected, i-FABP transcripts were undetectable in 48 hpf embryos (Fig. 4B) but present within the gut at 96 hours. Sectioning of the whole mount 96 hpf larvae showed that i-FABP, zRDHA and zRDHB transcripts were localized to epithelial cells (Fig. 4B). Interestingly, zRDHA and zRDHB transcripts showed sub-cellular localization in the microvillus brush-border region of the gut enterocytes. This finding is consistent with the sub-cellular localization of other transcripts within enterocyte brush-border microvilli (25).

We also performed rt-PCR analysis of zRDHA and zRDHB on RNA harvested from heart, skeletal muscle, eye, brain, fin, gut, and skin of 12 month old wildtype zebrafish. As a positive control for gut specific expression, we monitored the expression of the caudal-related transcription factor cdx1 (26). As expected, cdx1 showed strong expression that was confined to gut tissues in adult zebrafish (Fig. 4C). The expression of zRDHB, for the most part, paralleled that of cdx1 and showed highest expression levels in gut (Fig. 4C). In contrast, zRDHA signals were detected in all tissues examined, including gut (Fig. 4C). Furthermore, a survey of adult zebrafish intestines that had been dissected into six segments along the anterior-posterior axis revealed zRDHB expression predominantly in the anterior intestine. This was unlike zRDHA expression, which appeared constant along the gut AP axis (Fig. 4C).

**Knockdown of zRDHB phenocopies APC knockdown**

Because of the gut restricted expression of zRDHB in adults, we sought to abrogate the production of zRDHB protein in an effort to determine whether it functions as an RDH *in vivo* and, if so, whether it plays a critical role downstream of APC. To this end, we designed a splice-blocking morpholino that efficiently reduced zRDHB splicing when injected into embryos at the one-to-two cell stage (Fig. 5A). The zRDHB morphant fish displayed a number of phenotypes that recapitulated the APC morphants and that were absent from control morpholino-injected fish. These phenotypes
included pericardial edema (Fig. 5B), lack of jaw bone development (Fig. 5C) and failure to form pectoral fins (Fig. 5C).

The penetrance of each of the observed phenotypes depended on the amount of zRDHB morpholino injected and reached approximately 75% (n=152) in embryos injected with 0.5 nL of 0.5 mM antisense oligonucleotide. Animals that received this injection amount retained at least 50% viability. Higher injection amounts increased phenotype penetrance, but also caused severe mortality suggesting that further knockdown of zRDHB was lethal (Fig. 7E). This lethality was not observed in animals injected with identical amounts of a control morpholino. The similarities between phenotypes evoked by zRDHB knockdown and those reported for the zebrafish raldh2 genetic mutant known as neckless, strongly support zRDHB as an in vivo retinol dehydrogenase (15).

As seen with rescue of these structures in the APC morphants, we found that addition of retinoic acid rescued both fin and jaw phenotypes following zRDHB knockdown. Pectoral fin development was restored in 80% (n=92) of morphant embryos following retinoic acid treatment (Fig. 5C). Retinoic acid treatment partially rescued jaw formation in a similar proportion of zRDHB morphants as evidenced by alcian blue staining of developing jaw bones (Fig. 5C). In each case, treatment of morphant embryos with vehicle (DMSO) had no effect (Fig. 5C).

Similar to the APC morphants, we also noted pancreatic defects in the zRDHB morphant animals. Although histological analysis showed the presence of pancreatic structures in 96 hpf morphant fish (data not shown), in situ hybridizations using probes for trypsin (n=39) and insulin (n=32) demonstrated that approximately 80% of zRDHB morphants lacked mature pancreas (Fig. 5C). As seen with jaw and fin structures, treatment of morpholino-injected embryos with exogenous retinoic acid rescued the expression of both trypsin (64%, n=25) and insulin (67%, n= 24) (Fig. 5C).

**APC and zRDHB morphant zebrafish display intestinal differentiation abnormalities**

Since APC and zRDHB appeared to be required for the development of known, retinoic acid-dependent structures, we also examined knockdown embryos for intestinal defects. We observed that
APC and zRDHB morphants developed intestinal tubes normally (Fig. 6A) (22,23). For example, the anterior and posterior portions of gut fused correctly and the number of cells forming the perimeter of the intestine remained constant between control and morphant fish. However, the endodermal cells present in the APC and zRDHB morphant guts at 96 hpf appeared more characteristic of cells present in wildtype embryos at 72 hpf (Fig. 6A). Specifically, these cells remained cuboidal, rather than columnar, (Fig. 6A) and failed to express i-FABP (Fig 6B). These phenotypes are similar to those seen in APC mutant zebrafish (19) (personal communication, Anna-Pavlina Haramis). The penetrance of these phenotypes were similar in APC (73%, n=33) and zRDHB (76%, n=51) morphants.

Given the anterior distribution of zRDHB in adults and 96 hpf embryos (Fig. 4B and 4C), we sectioned and stained APC and zRDHB morphants along the AP axis (Fig. 6A). While knockdown of APC resulted in differentiation defects throughout the gut tube, we observed that zRDHB-dependent defects were confined to the anterior gut (Fig. 6A). Interestingly, sections from the midgut displayed an intermediate phenotype whereas the hindgut appeared to be unaffected by knockdown of zRDHB. Supporting a role for zRDHB downstream of APC, rt-PCR analysis of zRDHB levels in APC morphant embryos showed a 6.38 ± 0.42 fold decrease (mean ± S.D.) relative to wildtype embryos.

Rescue of intestinal structures under the retinoic acid treatment regimen used above resulted in re-expression of i-FABP (Fig. 6B) but did not result in full development of columnar cells (data not shown). Our data on the cell specific regulation of zRDHB suggests that full phenotypic rescue may require continuous exposure to retinoic acid in spatially distinct regions of the embryo. We could not achieve conditions necessary for full rescue because continuous exposure of whole embryos to retinoic acid led to numerous, non-gut developmental defects and was highly toxic to the embryos (data not shown).

**Expression of Hoxc8 depends on zRDHB and rescues aspects of zRDHB morphants**

Retinoic acid is known to play an important role in the timing of hox gene expression (27). We, therefore, considered the possibility that hox gene expression may be affected by knockdown of
zRDHB. Since, zebrafish *hoxc8* expression is present in the developing zebrafish pancreas, fin and spinal cord (http://zfin.org, 2003), we sought to determine whether *hoxc8* expression was affected by zRDHB knockdown. Following injection of zRDHB morpholino, we performed *in situ* hybridizations with a probe specific for *hoxc8*. Compared to wildtype embryos, zRDHB morphants expressed decreased levels of *hoxc8* through the head and spinal cord (Fig. 7A). In addition, *hoxc8* expression was lost in the fins and gut structures following zRDHB knockdown (Fig. 7A). Treatment of zRDHB morphants with retinoic acid concentrations that rescued structures within the zRDHB morphants also caused increased *hoxc8* expression in the head, spinal cord and gut structures (Fig. 7A). Further, compared to control injected embryos, low levels of *hoxc8* were ectopically induced by retinoic acid in regions of the spinal cord and tail (Fig. 7A). Finally, quantitative rt-PCR analysis confirmed the *in situ* hybridization patterns by demonstrating a reduction of *hoxc8* transcript levels in zRDHB morphant embryos and corresponding increase following retinoic acid treatment (Fig. 7B).

If zRDHB production of retinoic acid controls expression of *hoxc8* then the developmental appearance of *hoxc8* should parallel or follow expression of zRDHB. To examine this, we performed rt-PCR using primers specific for *hoxc8* on the same developmental stages utilized for assessment of zRDHB expression in Figure 4A. *Hoxc8* expression was detectable in embryos starting as early as six hours and was maximal by ten hours post-fertilization (Fig. 7C). The appearance of *hoxc8* corresponded directly with appearance of zRDHB (Fig. 4A).

In light of the data that *hoxc8* expression is dependent on zRDHB and retinoic acid, we considered the possibility that *hoxc8* overexpression might rescue some aspects of the zRDHB morphant phenotype. To test this hypothesis, we co-injected the zRDHB morpholino and *hoxc8* mRNA into one-to-two cell stage zebrafish embryos. We found that injection of *hoxc8* rescued pectoral fins (73%, n=101), insulin (76%, n= 21), trypsin (68%, n=22) and i-FABP (86%, n=28) indicating that *hoxc8* was sufficient to rescue zRDHB knockdown (Fig. 7D). Injection of mRNA encoding GFP showed no effect in rescuing embryonic defects (data not shown). Consistent with a role for hox genes
downstream of APC injection of hoxc8 mRNA into APC morphant provided rescued similar to that seen with the zRDHB morphants (Fig 7D).

We had found above that injection of embryos with high concentrations of zRDHB MO resulted in severe embryonic lethality. To examine the requirement of hoxc8 loss in this lethality, we injected embryos with lethal concentrations of the zRDHB morpholino along with hoxc8 mRNA. Co-injection of GFP mRNA served as a non-specific control and failed to rescue the zRDHB morpholino-injected embryos (Fig. 7E). In contrast, co-injection with hoxc8 mRNA led to a significant increase in embryo viability (Fig. 7E).
Discussion

Previous findings lead us to propose a model wherein APC controls retinoic acid production as a mechanism for controlling colonocyte differentiation. In the present study, we provide genetic evidence confirming a dependence of APC on retinoic acid production in regulating zebrafish development and enterocyte differentiation. In addition, our studies define a critical role for hox genes as signaling components downstream of APC and retinoic acid. Furthermore, we show that a retinol dehydrogenase is essential and rate-limiting in the normal development of a vertebrate.

We noted that phenotypes, including lack of jaw, pectoral fins and differentiated pancreas, of APC and zRDHB morphant embryos described in this report were very similar to those reported for the zebrafish raldh2 mutant, neckless, and a recently described APC mutant zebrafish (15,19). This observation supports a model wherein APC relies on retinoic acid biosynthesis during development, a notion that was confirmed by the rescue of APC morphant phenotypes with retinoic acid treatment. However, an interesting feature of the APC morphants is that they showed no anterior axis defects. APC is known to negatively regulate β-catenin and, therefore, antagonize WNT signaling. Consistent with this, we found that the β-catenin targets c-myc and cyclin D were elevated in APC morphants suggesting loss of canonical APC function. There appears, however, to be a discrepancy between knockout of APC and introduction of β-catenin. β-catenin injection causes axis duplication in both zebrafish (28) and Xenopus (29,30). Knockdown of APC in zebrafish, however, does not appear to directly recapitulate overexpression of β-catenin. This may be explained by the presence of maternally supplied APC in the APC knockdown embryos in that use of an APC-directed splicing blocking morpholino targets only zygotic APC transcripts. Maternally provided APC may have afforded enough control on β-catenin to prevent axis duplication. If so, this maternally supplied APC was apparently insufficient to prevent the robust retinoic acid-dependent APC morphant phenotypes, thereby suggesting an APC-retinoic acid link that is independent of β-catenin. Perhaps regulation of retinoic acid biosynthesis by APC complements its de-stabilization of β-catenin. Indeed, recent studies indicate
that retinoic acid inhibits the transcriptional activity of β-catenin (31) and promotes the translocation of β-catenin from the cytoplasm to the membrane (32).

In support of a role for APC and retinoic acid in enterocyte differentiation, we found that knockdown of APC or zRDHB also caused intestinal defects. Cells within APC and zRDHB morphant guts remained cuboidal rather than columnar and failed to express i-FABP. Confirmation of the role for retinoic acid in gut development and differentiation was provided by the rescue of i-FABP expression following treatment of zRDHB morphants with exogenous retinoic acid. The gut tubes of APC and zRDHB morphant embryos, despite their lack of differentiated characteristics, were in some respects fully formed at 96 hpf and resembled the guts in wildtype embryos at 72 hpf. At 72 hpf wildtype gut tubes have formed with defined endoderm and mesoderm. The endoderm at this point, however, lacks evidence of differentiation suggesting a requirement for retinoic acid following 72 hpf. The APC and zRDHB morphant gut phenotypes are, therefore, consistent with previous reports by Stafford et al. showing that treatment of zebrafish embryos with a pan-retinoic acid antagonist caused no defects in gut tube formation in embryos up to 72 hpf (17). These studies did not examine the gut tube or epithelial cell morphology at time points beyond 72 hpf (17). Taken together the current data indicate a requirement for retinoic acid production in enterocytes starting between 72 and 96 hpf and place retinoic acid production as a critical requirement in the transition of early gut endoderm into differentiated epithelial cells. This temporal requirement highlights the need for precise timing of zRDHB expression and subsequent retinoic acid production. Indeed, we have been unable to detect zRDHB, or zRDHA, expression in the zebrafish gut prior to 72 hpf (data not shown).

Despite some data implicating retinoic acid in intestinal cell functions, vitamin A deficiency does not appear to cause complete loss of intestinal epithelial differentiation in adult rats (33-37). This is in contrast to the data presented, here, in which APC or zRDHB morphant zebrafish embryos showed dramatic suppression of gut epithelial differentiation. The discrepancy between rodent models of vitamin A deficiency and knockout in zebrafish embryos may be explained in a number of ways. First, it
may indicate that retinoic acid plays an important role in early development of the gut, but plays a lesser role in gut maintenance in adults. It is also possible, however, that vitamin A deficiency regimes do not fully deplete intestinal stores of vitamin A and, therefore, do not result in a complete suppression of retinoic acid production. Genetic knockout of gut-specific retinol biosynthesis in rodents may provide additional evidence to clarify the contribution of retinoic acid to intestinal epithelial cell differentiation in adults.

Knockout of zRDHB in zebrafish offers an important new observation regarding the hierarchy of retinoid biosynthetic enzymes \textit{in vivo}. Recent work in mice has raised a question as to which enzymes, retinol dehydrogenases or retinal dehydrogenases, are essential and rate-limiting for retinoic acid production \textit{in vivo}. Indeed, knock out of several murine retinol dehydrogenases including ADH1, ADH4 and RDH5 has resulted in mice with only mild phenotypes and normal tissue capacity for retinoic acid production (38-40). In contrast, knockout of retinaldehyde dehydrogenases, such as RALDH2, resulted in substantial reductions in retinoic acid production and caused embryonic lethality (41). This has led to a model wherein retinol dehydrogenases are ubiquitous and redundant. Recent studies have shown that ADH3 may function as a ubiquitous retinol dehydrogenase in mouse tissue, thus explaining the lack of phenotypes seen in previous knockout experiments. The redundancy of ADHs, therefore, points to retinaldehyde dehydrogenases as the tissue specific regulators of retinoic acid production. However, knockdown of zRDHB in zebrafish resulted in severe, tissue specific developmental abnormalities that, for the first time, illustrate an essential role of a single retinol dehydrogenase \textit{in vivo}. This essential function of zRDHB is consistent with the tissue specific nature of zRDHB in that zRDHB appears confined to the anterior gut in zebrafish after 72 hpf. Targeted abrogation of zRDHB splicing had an effect only on the anterior portion of the developing intestine. In contrast, knockdown of APC resulted in defects throughout the gut. In fact, we found that zRDHA was expressed in all tissues and all regions of the gut in adult zebrafish. This raises the possibility that some RDHs, like zRDHA, may serve to establish a basal level of retinoic acid production in tissues that additional RDHs, like zRDHB, complement as needed. This would create a mechanism to establish gradients of retinoic acid
production and would complement the known requirement for the retinoic acid inactivating P-450 enzyme, CYP-26, in maintaining a retinoic acid gradient needed to define anterior versus posterior development (42,43).

Hoier et al. have recently reported that the APC-like gene apr-1 directs Hox gene expression during C. elegans embryogenesis and vulval development (44). Our data support a role for hox genes downstream of APC and retinoic acid in that hoxc8 was able to rescue APC and zRDHB morphant phenotypes. Given the expression boundaries of hoxc8 compared to the tissues affected by APC or zRDHB knockdown, hoxc8 is unlikely to be the only hox gene linked to APC function. For example, hoxc8 mRNA injection rescued expression of i-FABP despite the low to undetectable levels of hoxc8 in the intestines of wildtype embryos (data not shown). These findings are consistent with recent studies in zebrafish demonstrating hox gene redundancy in rescuing zebrafish blood progenitors (45). In view of these observations, it is important to consider that APC and retinoic acid may target additional hox genes within the embryo and within different regions of the gut.

It was surprising that hoxc8 was able to rescue retinoic acid deficient phenotypes in the zRDHB morphants. We anticipated that retinoic acid would have a much broader target spectrum that would not be efficiently duplicated by co-injection with any single downstream target gene. This indicates that retinoic acid transcriptional targeting may be more narrow than expected and may in fact rely on tissue specific transcriptional activators that remodel target chromosomal regions. Consistent with this, Perz-Edwards et al. have reported that a transgenic zebrafish carrying a synthetic retinoic acid response promoter coupled to GFP showed limited activation within the fish (46). Notably they did not report activation of this construct within the gut but rather saw strong activation in the developing neural tissues. These data indicate that tissue-specific control of retinoic acid responsiveness is maintained at several levels. First, it must be governed by activation and expression of retinoic acid biosynthetic genes, and second, it must rely on tissue-specific transcription factors for correct targeting of tissue-specific retinoic acid target genes.
The reliance of APC on retinoids in fish harbors important implications for human disease given that APC is mutated in as many as 85% of colon carcinomas. Since APC induced the expression of human RDHL and increased retinoic acid production in colon cancer cell lines (10), it is likely that regulation of retinoic acid production by APC is conserved between zebrafish and humans. Loss of APC in human colon tumors, therefore, would predict the absence of retinol dehydrogenase as an early event in formation of colon adenomas and carcinomas. Indeed, human RDHL expression levels were reduced in 70% of colon adenomas and carcinomas examined (10). Taken together, the existing data raise the possibility of preventing colon adenoma formation through pharmacologic restoration of retinoid activity.
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References


Figure Legends

Figure 1. *Morpholino knock-down of APC causes developmental defects that are rescued by retinoic acid.* (A) Rt-PCR with primers targeted to intron and exon sequences within zebrafish APC distinguished un-spliced from spliced APC transcripts following injection of embryos with a control or splice blocking APC morpholino and confirmed APC knockdown. (*lane 1, control morpholino; lane 2, control morpholino with no rt; lane 3, APC morpholino; lane 4, APC morpholino with no rt.*) Amplification of $\beta$-actin confirmed equal amounts of input cDNA (*lower panel*). (B) Quantitative rt-PCR with primers specific for $\beta$-actin (*bar 1*), c-myc (*bar 2*), and cyclin D1 (*bar 3*) was performed on cDNAs obtained from embryos injected with control or APC morpholinos. Fold increases for each target transcript were determined by comparing the number of transcript per $\mu$g total RNA in APC morpholino-injected embryos to the number of transcript per $\mu$g total RNA in control morpholino-injected embryos. Shown are mean values $\pm$ S.D. (C) Pectoral fins and jaws (alcian blue staining) were absent in embryos (96 hpf) following injection of an APC morpholino (*APC MO*). Injection of equal amounts of control morpholino (*Con MO*) showed no effects on pectoral fin or jaw formation. Treatment of APC morphants with retinoic acid (*RA*) rescued both fin and jaw formation whereas vehicle treatment (*center panels*) did not. (D) Whole-mount *in situ* hybridizations with probes for insulin and trypsin indicated that embryos (96 hpf) injected with an APC morpholino (*APC MO*) failed to develop endocrine and exocrine pancreas in comparison to embryos injected with a control morpholino (*Con MO*). Retinoic acid treatment rescued expression of insulin and trypsin in the APC morphant embryos whereas treatment with vehicle (*center panels*) failed to do so.

Figure 2. *Alignment of the novel zebrafish retinol dehydrogenases, zRDHA and zRDHB, with human RDHL.* Human RDHL was used to search all zebrafish EST databases and led to identification of two novel zebrafish retinol dehydrogenases. An alignment of zRDHA, zRDHB and hRDHL showed that zRDHA and zRDHB were 47.0% and 45.5% identical to hRDHL respectively. Identical residues are shaded in black and conserved catalytic regions indicated by a dotted underline.
Figure 3. zRDHA and zRDHB align with retinol dehydrogenases and facilitate conversion of retinol into retinoic acid. (A) A phylogenetic analysis placed zRDHA and zRDHB within a larger family of known retinol dehydrogenases. GenBank accession numbers are provided for each aligned sequence. (B) zRDHA and zRDHB were over-expressed with a N-terminal V5-tag in the human colon cell line, HCT116. Cells carrying empty vector (bar 1), V5-zRDHA (bar 2) or V5-zRDHB (bar 3) were incubated with 100 nmol of retinol for 18 hours. Retinooids were then extracted from the culture medium using acidified hexane and separated by RP-HPLC. Retinoic acid levels were quantified by comparison to the internal extraction standard, TTNP. Data shown are mean ± S.D.

Figure 4. Expression analyses of zRDHA and zRDHB in adult and developing zebrafish. (A) Developmental expression of zRDHA, zRDHB, Cdx1 and β-actin was examined by rt-PCR in cDNA derived from embryos at various developmental stages. (Lane 1, size standards; lane 2, 0 hpf; lane3, 6 hpf; lane 4, 10 hpf; lane 5, 24 hpf; lane 6, 48 hpf; lane 7, 72 hpf; lane 8, 96 hpf; lane 9, 120 hpf; lane 10, no input). (B) Whole-mount in situ hybridization on 48 and 96 hpf embryos using antisense digoxigenin-labeled RNA probes for neomycin, i-FABP, zRDHA and zRDHB show redistribution of zRDHA and zRDHB expression from the head regions to the gut. Histologic cross-sections of 96 hpf embryos following whole mount in situ hybridization show confinement of zRDHA and zRDHB to gut epithelial cells. (C) Expression levels of zRDHA and zRDHB were determined by rt-PCR in adult zebrafish tissues (left panel). Lane 1, molecular weight marker; lane 2, adult heart; lane 3, skeletal muscle; lane 4, eye; lane 5, brain; lane 6, fins; lane 7, gut; lane 8, skin; lane 9, no input. Cdx1 was utilized as a known gut specific transcription factor. β-actin confirmed equal amounts of cDNA input. A similar analysis was performed on adult zebrafish intestines that had been sectioned into six segments (lane 2, most anterior; lane 8, most posterior) along the antero-posterior axis (right panel).
Figure 5. **Morpholino knockdown of zRDHB phenocopies knockdown of APC and is partially rescued by retinoic acid.** (A) Rt-PCR analysis using intron spanning primers indicated that embryos injected with a control morpholino (*lane 2*) expressed fully spliced zRDHB transcripts whereas embryos injected with a zRDHB morpholino showed loss of spliced and gain of unspliced zRDHB transcripts (*lane 4*). Reactions without reverse transcriptase confirmed amplification from cDNA (*lanes 3 and 5*). (B) The gross phenotype of a typical zRDHB morphant compared to a control injected larva. (C), Comparison of control morpholino (*Con MO*) or zRDHB morpholino (*zRDHB MO*) injected embryos at 96 hpf revealed that zRDHB morphants lacked pectoral fins and jaws. In addition, zRDHB morphants failed to express insulin and trypsin as assessed by whole-mount *in situ* hybridization. Treatment of zRDHB morphant embryos with either vehicle (*center panels*) or retinoic acid (*RA*) showed that retinoic acid partially restored formation of pectoral fins and jaws as well as expression of insulin and trypsin.

Figure 6. **APC and zRDHB morphants display intestinal defects that are rescued by retinoic acid.** (A) Control, APC and zRDHB morphants (96hpf) were sectioned and stained with H&E. APC and zRDHB morphants lacked differentiated columnar epithelial cells and showed an epithelial morphology similar to that present in the undifferentiated guts of wildtype embryos at 72 hpf. (B) Whole mount *in situ* hybridization with a probe specific to the enterocyte differentiation marker i-FABP confirmed an absence of differentiation as seen in the morphological examination. Treatment of APC and zRDHB morphant embryos (96 hpf) with retinoic acid (*RA*) restored levels of i-FABP within the gut.

Figure 7. **Injection of hoxc8 mRNA rescues zRDHB morphant phenotypes.** (A) Whole-mount *in situ* hybridization determined the distribution of *hoxc8* gene expression within 48 hpf embryos that had been injected with a control (*Con MO*) morpholino. A zRDHB (*zRDHB MO*) morpholino reduced hybridization of the *hoxc8* probe in the head and gut regions. Treatment of zRDHB morphants with retinoic acid (*RA*) restored *hoxc8* expression levels. (B) *Hoxc8* transcript levels in zRDHB morphants (*bar 1*) and zRDHB morphants treated with retinoic acid (*bar 2*) were determined by rt-PCR. Values
shown are mean ± S.D. (C) Developmental expression of hoxc8 and β-actin was examined by rt-PCR in cDNA derived from embryos at various developmental stages. (Lane 1, size standards; lane 2, 0 hpf; lane 3, 6 hpf; lane 4, 10 hpf; lane 5, 24 hpf; lane 6, 48 hpf; lane 7, 72 hpf; lane 8, 96 hpf; lane 9, 120 hpf; lane 10, no input). Hoxc8 expression follows the appearance of zRDHA and zRDHB. (D) Full-length hoxc8 RNA was transcribed and co-injected along with zRDHB MO or APC MO into wildtype zebrafish embryos. Following injection, embryos (96 hpf) were analyzed visually for the presence of pectoral fins and in situ hybridizations were performed to monitor the expression of trypsin, insulin and i-FABP. (E) Wildtype zebrafish embryos were injected with concentrations of zRDHB MO sufficient to cause lethality. Co-injection of GFP mRNA or hoxc8 mRNA was performed to determine whether hoxc8 could reverse lethality. The percent survival of zRDHB morphant embryos was calculated by counting the number of viable embryos at 96hpf compared to the total number initially injected. (Bar 1, zRDHB MO + GFP mRNA; bar 2, zRDHB MO + hoxc8 mRNA, bar 3, Con MO + GFP mRNA).
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