Mutational mechanisms that activate Wnt signaling and predict outcomes in colorectal cancer patients

William Hankey[1], Michael A. McIlhatton[1], Kenechi Ebede[2], Brian Kennedy[3], Baris Hancioglu[3], Jie Zhang[4], Guy N. Brock[3], Kun Huang[4] and Joanna Groden[1 ‡]

[1]Department of Cancer Biology and Genetics, College of Medicine, The Ohio State University, Columbus, OH 43210
[2]Department of Anesthesiology, University of Florida, Gainesville, FL 32603
[3]Department of Biomedical Informatics, College of Medicine, The Ohio State University, Columbus, OH 43210
[4]Department of Medical and Molecular Genetics, Indiana University, School of Medicine, Indianapolis, 46202

‡ Corresponding Author
Address: 986 BRT 460 W. 12th Ave. Columbus, OH 43210.
Phone: (614) 688-4301
E-mail: groden.2@osu.edu

Running Title: Transcriptional changes unique to *Apc*-mutant colon adenomas

The authors declare no potential conflicts of interest.

This is the author's manuscript of the article published in final edited form as:

Hankey, W. C., McIlhatton, M. A., Ebede, K., Kennedy, B., Hancioglu, B., Zhang, J., … Groden, J. L. (2017). Mutational mechanisms that activate Wnt signaling and predict outcomes in colorectal cancer patients. Cancer Research, canres.1357.2017. https://doi.org/10.1158/0008-5472.CAN-17-1357

Abstract

APC biallelic loss-of-function mutations are the most prevalent genetic changes in colorectal tumors, but it is unknown whether these mutations phenocopy gain-of-function mutations in the *CTNNB1* gene encoding ß-catenin that also activate canonical WNT signaling. Here we demonstrate that these two mutational mechanisms are not equivalent. Further, we show how differences in gene expression produced by these different mechanisms can stratify outcomes in more advanced human colorectal cancers. Gene expression profiling in *Apc*-mutant and *Ctnnb1*-mutant mouse colon adenomas identified candidate genes for subsequent evaluation of human TCGA data for colorectal cancer outcomes. Transcriptional patterns exhibited evidence of activated canonical Wnt signaling in both types of adenomas, with *Apc*-mutant adenomas also exhibiting unique changes in pathways related to proliferation, cytoskeletal organization and apoptosis. *Apc*-mutant adenomas were characterized by increased expression of the glial nexin Serpine2, the human ortholog of which was increased in advanced human colorectal tumors. Our results support the hypothesis that *APC*-mutant colorectal tumors are transcriptionally distinct from *APC*-wild-type colorectal tumors with canonical WNT signaling activated by other mechanisms, with possible implications for stratification and prognosis.

Introduction

Personalized medicine has permitted stratification of tumors into subtypes distinguishable by molecular characteristics associated with different prognoses or therapeutic responses.

In colorectal cancer, activating mutations in the *KRAS* gene, for example, correlate strongly with poor prognosis (1) and guide therapeutic decision-making (2). Development of additional markers is needed, however, to define stage-specific characteristics associated with clinical outcomes.

The most prevalent genetic changes in colorectal tumors are biallelic *APC* mutations; although they have an unclear relationship to outcome (3), they drive dramatic changes in both gene expression and cellular phenotype. We asked whether biallelic loss-of-function *APC* mutations are equivalent to gain-of-function mutations in the *CTNNB1* gene encoding β-catenin, as both mutations activate canonical WNT signaling and occur in a mutually exclusive fashion (4). Mutational data show that *CTNNB1* mutations become less prevalent in the tumor spectrum from small adenomas to large adenomas and adenocarcinomas, while *APC* mutations remain well-represented (5). This difference in prevalence may be explained by WNT-independent functions of the APC tumor suppressor protein for which loss significantly contributes to colorectal tumor progression (6-15), and retention in *CTNNB1*-mutant tumors may impair progression.

Nuclear APC and chromatin-associated APC interact with β-catenin to inhibit canonical WNT signaling, but may mediate β-catenin-independent transcriptional changes as well. Previous transcriptional profiling studies of human and murine colon tumors have assumed the equivalence of *APC* and *CTNNB1* mutations in their effects on gene transcription. Our previous work described a transcriptional profiling of adenomas from four murine models of intestinal cancer, focused on the shared characteristics of activated canonical WNT signaling between colon tumors from $Apc^{Min/+}$ mice and azoxymethane-

and dextran sulfate sodium- (AOM/DSS) treated mice now known to harbor *Ctnnb1* mutations (16,17). The transcriptional evidence of activated canonical WNT signaling in these *Apc*- and *Ctnnb1*-mutant tumors stood out in comparison to colon adenomas from mouse models with aberrant Smad/TGFβ1 signaling (16,17). Subtle differences between *APC* and *CTNNB1* mutant colorectal tumors were not examined.

This study tests the hypothesis that *APC*-mutant colon tumors exhibit a set of changes in gene expression that overlap with those in *CTNNB1*-mutant tumors, as well as a unique set of changes in gene expression due to the loss of chromatin-associated APC and absent from *CTNNB1*-mutant tumors where such binding is retained. These differences in gene expression patterns were first identified by a comparison of transcriptional profiles of mouse colon adenomas bearing either biallelic *Apc* mutations (from $Apc^{Min/+}$ mice) or an activating *Ctnnb1* mutation (from mice treated with AOM/DSS). Transcriptome profiling (mRNA-seq) of colon adenomas from each mouse model was performed to generate a list of up- and down-regulated transcripts for each type of adenoma. Transcriptional changes in *Apc*-deficient adenomas exhibited significant overlap with AOM/DSS adenomas, but also included unique changes related to the regulation of proliferation, apoptosis and cytoskeletal organization. Adenomas from $Apc^{Min/+}$ mice are characterized by increased expression of *Serpine2* in comparison to adenomas with *Ctnnb1* mutations. Expression of its human ortholog is directly correlated with stage in human colorectal tumors. Future studies will test the clinical utility of this candidate marker in identifying at-risk patients with potentially more advanced disease and will

characterize the relationship between its expression and chromatin-associated functions of APC.

Materials and Methods

Generation of $Apc^{Min/+}$ and AOM/DSS-treated mouse colon adenomas

Azoxymethane- and dextran sulfate sodium-treated (AOM/DSS) mice were treated as follows: 6-7 week old *C57BL/6J* mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight). One week later mice were treated with 2% DSS (in drinking water), followed by two weeks on water alone. They received 3 cycles of DSS / water. Mice were sacrificed seventy days after AOM was administered. $Apc^{Min/+}$ mice on a *C57BL/6J* background were sacrificed at 110-120 days old. Whole mouse colons were harvested from sacrificed $Apc^{Min/+}$ and AOM/DSS-treated mice and immediately stored in RNAlater solution (Thermo Fisher Scientific, catalog # AM7020) to protect RNA quality. Single adenomas were excised from the colons of each mouse, and an adjacent section of non-tumor colon tissue was taken as a control. The OSU Institutional Animal Care and Use Committee granted prior approval for all animal studies.

RNA isolation

Tissues were homogenized into 500 μL each of Trizol (Life Technologies catalog # 15596-026) using an OMNI TH tissue homogenizer over a period of 1-2 minutes each. 50 μg glycogen (RNase-free) was mixed into each sample. A standard Trizol isolation protocol was then performed (18) with one modification: Because the mixture appeared

cloudy following the addition of isopropanol (due to the precipitation of residual salts from the RNAlater solution), a 50% mixture of nuclease free water and isopropanol was added until the solution became clear. RNA was resuspended in nuclease-free water.

Library preparation and sequencing

Preparation of eight single-read libraries (from three colon adenomas and one adjacent non-adenoma colon tissue from $Apc^{Min/+}$ mice, and from three colon adenomas and one adjacent non-adenoma colon tissue from AOM/DSS-treated mice) was performed using the TruSeq RNA Library Preparation Kit (Illumina catalog # RS-122-2001). mRNA sequencing was performed by The Ohio State University Genomics Shared Resource using a HiSeq 2500 instrument (Illumina, Inc.). Data processing and analysis were performed by The Ohio State University Biomedical Informatics Shared Resource. The transcript expression levels were quantified in FPKM units. Data were normalized by dividing the FPKM measurement for each adenoma by that of its corresponding adjacent non-adenoma tissue. Differentially expressed transcripts between Apc-deficient ($Apc^{Min/+}$) and *Apc*-wild-type (AOM/DSS) colon adenomas were identified using the two-tailed Student's *t*-test to compare the fold-changes over non-adenoma tissue between three adenomas of each type. Adenomas from AOM/DSS-treated mice were confirmed to express degradation-resistant β-catenin as previously reported (19), indicating that they were *Apc*-wild-type (4). Sanger sequencing was done by reverse-transcribing an RNA sample from each adenoma from AOM/DSS-treated mice and from the adjacent non-adenoma sample using SuperScript II reverse transcriptase (Thermo Fisher Scientific,

catalog # 18064022). The *Ctnnb1* gene was then PCR-amplified using the primers *Ctnnb*1S (GCTGACCTGATGGAGTTGGA) and *Ctnnb1*AS (GCTACTTGCTCTTGCGTGAA), and Sanger sequenced from each side using the same primers. The point mutations observed were all between codons 32 through 41 and consistent with previous reports (19). The raw and processed mRNA-seq data are available through NCBI's Gene Expression Omnibus (accession number GSE98496, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wpidaoiuphunvcn&acc=GSE98496.)

In silico analysis of gene expression data from mouse colon tumors

Approximately half of the genes listed in the RNA-seq analysis (14,301 out of 31,189 total) were considered to have sufficient counts (an average > 32 across the 8 sequenced samples) to enable reliable expression analysis. FPKM measurements were used to calculate fold-changes for each adenoma relative to matched non-adenomatous colon tissue. 3,097 transcripts were differentially expressed by at least 2-fold (one-sample *t*-test, FDR < 0.1) in colon adenomas from $Apc^{Min/+}$ mice relative to the corresponding non-adenomatous colon tissue. 1,289 transcripts were differentially expressed by at least 2-fold (one-sample *t*-test, FDR < 0.1) in colon adenomas from AOM/DSS-treated mice relative to the corresponding non-adenoma colon tissue. Analysis of overlap between differentially expressed transcripts from both adenoma types identified 830 transcripts differentially expressed in the same direction in colon adenomas from $Apc^{Min/+}$ and AOM/DSS-treated mice. 139 of the transcripts identified in adenomas from AOM/DSS-

treated mice were differentially expressed between the two adenoma types (The Student's *t*-test, FDR < 0.05). 231 of the transcripts identified in adenomas from $Apc^{Min/+}$ mice were differentially expressed between the two adenoma types (The Student's *t*-test, FDR < 0.05).

Gene Ontology

Gene ontology analysis was performed using Ingenuity Pathway Analysis (IPA) software. 231 transcripts differentially expressed in adenomas from $Apc^{Min/+}$ mice, 139 transcripts differentially expressed in adenomas from AOM/DSS-treated mice, and 830 transcripts differentially expressed in both adenoma types relative to matched adjacent colon tissue were uploaded to IPA along with fold-changes in expression. Default settings were used, and transcripts not significantly changed in adenomas were omitted from the data submitted to IPA, in order to focus the analysis on statistically significant changes. Significantly enriched or depleted categories were tabulated and functional pathways predicted to be increased or decreased were indicated by red or blue color, respectively.

Filtering transcripts differentially expressed in adenomas from $Apc^{Min/+}$ mice using datasets from mouse models of canonical Wnt signaling inactivation

The lists of transcripts unique to each adenoma type were narrowed by the removal of those differentially expressed at least 1.5-fold in the other adenoma type relative to non-adenomatous tissue. Transcripts unique to adenomas from $Apc^{Min/+}$ mice were narrowed

from 231 to 148, while transcripts unique to adenomas from AOM/DSS-treated mice were narrowed from 139 to 92. Additional filtering of transcripts unique to adenomas from *Apc*$^{Min/+}$ mice was performed using two publicly available datasets profiling gene expression following either inactivation of the gene encoding β-catenin in the mouse intestine (20) or the inactivation of the canonical WNT signaling transcription factor TCF4/TCF7L2 in human colon cancer cells (21). The first dataset identified 1,697 transcripts up- or down-regulated by at least 1.25-fold following β-catenin inactivation in the mouse intestine, relative to the untreated control, enabling the removal of 14 overlapping transcripts from those unique to adenomas from *Apc*$^{Min/+}$ mice, reducing the number of genes of interest from 148 to 134. The second dataset identified 172 transcripts up-regulated in mouse adenomas and human colon cancer cell lines following introduction of a dominant negative TCF4/TCF7L2, enabling the removal of one additional overlapping transcript from those unique to adenomas from *Apc*$^{Min/+}$ mice, reducing the number of genes of interest from 134 to 133.

Filtering transcripts differentially expressed in adenomas from Apc$^{Min/+}$ *mice using APC ChIP-seq data*

Human orthologs were identified for 120 of the 133 genes differentially expressed in colon adenomas from *Apc*$^{Min/+}$ mice and for 81 of the 92 genes differentially expressed in colon adenomas from AOM/DSS-treated mice. These were compared with APC ChIP-seq data generated from the HCT-116 human colon cancer cell line. APC-associated genomic regions were identified by immunoprecipitation from the chromatin fraction of

these cells, followed by massively parallel sequencing (ChIP-seq), as described previously (22). ChIP-seq data identified 44,771 APC-associated genomic regions, of which 12,840 occurred within 10 kb upstream or downstream of a transcription start site, facilitating identification of a candidate target gene. 81 (68%) of the 120 genes differentially expressed in colon adenomas from *Apc*$^{Min/+}$ mice were encoded within 10 kb of an APC-associated genomic region. The relevant raw and processed ChIP-seq data are available through the NCBI Gene Expression Omnibus (accession number GSE98496, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wpidaoiuphunvcn&acc=GSE98496.)

In silico analysis of gene expression data from human colorectal tumors

Analysis of gene expression in human tumors was performed for 81 transcripts of interest differentially expressed in colon adenomas from *Apc*$^{Min/+}$ mice and encoded by genomic loci bound by chromatin-associated APC. Gene level RNA-seq data (in FPKM units) and clinical data for 357 human colorectal tumors of all stages were obtained in January of 2017 as part of the "TCGA Provisional" dataset (generated by the TCGA Research Network: http://cancergenome.nih.gov/) from the cBioportal website (23). Tumors were sorted by stage and expression of each gene was compared in early stage (I and II) vs. late stage (III and IV) tumors. Genes increased in expression in *Apc*$^{Min/+}$ adenomas relative to non-adenomatous tissue were hypothesized to increase in expression from early to late stage tumors. Eleven such genes increased significantly in expression in

Stage III and IV tumors relative to Stage I and II tumors (one-tailed Student's *t*-test, FDR < 0.05), while one such gene decreased significantly (FDR < 0.05) instead. These twelve genes were similarly examined in gene expression data from 242 human colorectal tumors of all stages obtained in January of 2017 as part of the TCGA dataset (generated by the TCGA Research Network: http://cancergenome.nih.gov/) from the cBioportal website. The *MACC1* gene was a useful control known to increase in expression in more advanced tumors (24).

Quantitative PCR validation in an independent set of mouse colon adenomas

RNA-seq results were validated for twelve individual transcripts of interest using independent sets of colon adenomas and non-tumor colon tissues from each mouse model. RNA was isolated from three colon adenomas and three adjacent non-tumor colon tissue samples from three different $Apc^{Min/+}$ *C57BL/6J* mice, and from three colon adenomas and three adjacent non-tumor colon tissues samples from a single AOM/DSS-treated *C57BL/6J* mouse. cDNA was reverse transcribed from 1 μg of total RNA as previously described, and 100% conversion to cDNA was assumed for calculation purposes. qPCR was performed in 20-μL reactions with final concentrations of 300nM of each primer and 10 ng of total cDNA, using Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sets were designed to span exon junctions (whenever possible) and were confirmed to amplify cDNA with a single melting temperature.

Analysis of gene expression as a potential marker for disease-free survival

Clinical data measuring time to recurrence or death was available for the 357 tumors from the "TCGA Provisional" dataset, provided in the "Disease-Free (Months)" category, or in the "Overall Survival (Months)" category when the former was not available. Tumors were sorted into two groups for each transcript of interest, based on expression levels higher or lower than the median. Kaplan-Meier curves were constructed comparing disease-free survival in the high-expression and low-expression subgroups, and statistical significance was evaluated using the Cox-Mantel Log-Rank test (FDR < 0.05). A similar validation analysis was performed using 242 tumors from the "TCGA" dataset.

Analysis of gene expression as a potential marker for APC mutational status

Genes increased in expression in $Apc^{Min/+}$ adenomas relative to non-adenomatous tissue were hypothesized to increase in expression in *APC* mutant human tumors. Gene expression was examined in 242 tumors from the "TCGA" dataset, of which 203 had been sequenced at the *APC* locus. Tumors were divided into two subgroups of 55 "*APC* wild-type tumors" (including those with missense mutations) and 148 '*APC* mutant tumors" (including those with frameshift mutations or other premature stop codons detected in either or both alleles of *APC*). Statistical significance was evaluated using one-tailed Student's *t*-test (FDR < 0.05). The *CCND1*, *MYC* and *MACC1* genes were similarly evaluated as controls.

Results

Transcriptional profiling of colon adenomas and adjacent colon tissues

This study tests the hypothesis that chromatin-associated APC protein regulates the transcription not only of canonical WNT target genes but also that of WNT/β-catenin-independent target genes. Initial experiments were designed to identify transcripts altered in *Apc*-mutant colon adenomas but not in *Ctnnb1*-mutant colon adenomas in which β-catenin is rendered resistant to degradation with chromatin-associated Apc functions intact. mRNA sequencing (mRNA-seq) was performed to profile gene expression in colon adenomas with *Apc* mutations (from $Apc^{Min/+}$ *C57BL/6J* mice) and colon adenomas with *Ctnnb1* mutations (from AOM/DSS-treated *C57BL/6J* mice) in comparison to adjacent non-adenoma tissue from each mouse model. Previous studies of colon adenomas from the AOM/DSS model reported the occurrence of activating mutations in *Ctnnb1* (19). PCR and Sanger sequencing confirmed changes in codons 32 and 33 of *Ctnnb1* in the AOM/DSS adenomas used here (data not shown). The presence of *Ctnnb1* mutations indicates that the adenomas are wild-type for *Apc* (4).

Existing literature has established histopathological similarities between the two adenoma models, including shared dysplastic changes such as nuclear overlap and crowding, hyperchromatic nuclei and high nucleocytoplasmic ratio (16). Colon adenomas from AOM/DSS-treated mice develop in the context of colitis and are associated with surrounding and infiltrating mast cells, while colon adenomas from $Apc^{Min/+}$ mice exhibit less inflammation (25). These known similarities and differences between colon adenomas from $Apc^{Min/+}$ *versus* AOM/DSS-treated mice informed our interpretations of RNA-seq data. Differential gene expression analysis was performed using RNA-seq to

calculate fold-changes for colon adenomas from *Apc*$^{Min/+}$ or AOM/DSS-treated mice relative to matched non-tumor colon tissue controls. Transcripts that changed by at least 2-fold (one-sample *t*-test, FDR < 0.1) were identified for AOM/DSS-derived and *Apc*$^{Min/+}$-derived adenomas (Fig. 1). Of the 3,097 transcripts differentially expressed in colon adenomas from *Apc*$^{Min/+}$ mice and 1,289 transcripts differentially expressed in colon adenomas from AOM/DSS-treated mice, 830 overlapping transcripts were differentially expressed in the same direction in both colon adenoma types relative to their respective non-adenoma controls. These overlapping transcripts included *Myc* and other known targets of the canonical WNT signaling pathway. The high FDR cutoff value for this initial step allowed the majority of these internal controls to pass the initial screening step. Fold-changes from the two adenoma types were then compared to identify 231 transcriptional changes unique to adenomas from *Apc*$^{Min/+}$ mice and 139 transcriptional changes unique to adenomas from AOM/DSS-treated mice (Student's *t*-test, FDR < 0.05). This step and the subsequent screening process are illustrated in Figure 1.

Initially, thirty-five of the best known target genes of the canonical Wnt signaling pathway were examined as internal controls. Twenty of these transcripts (Fig. 2) changed in expression in at least one type of adenoma relative to matched non-tumor tissue. Eighteen of these transcripts changed in expression by at least two-fold both in adenomas from AOM/DSS-treated mice and in those from *Apc*$^{Min/+}$ mice. The two remaining transcripts, *Birc5* and *Ephb3*, increased by more than 2-fold in adenomas from *Apc*$^{Min/+}$ mice but by less than 1.5-fold in adenomas from AOM/DSS-treated mice. Both

types of adenomas exhibited strong evidence of activated canonical WNT signaling relative to their adjacent non-adenoma tissues. Adenomas from *Apc*$^{Min/+}$ mice exhibited greater activation of certain WNT targets such as *Birc5, Ephb3, Axin2* and *Sox17*.

Gene ontology

231 transcriptional changes unique to adenomas from *Apc*$^{Min/+}$ mice, 139 unique to AOM/DSS-treated mice, and 830 shared by both adenoma types relative to matched adjacent colon tissue were subjected to gene ontology using Ingenuity Pathway Analysis software (IPA) from QIAGEN (Fig. 3A-C). Transcript names were uploaded along with associated fold-changes in expression. Transcripts changing only in adenomas from AOM/DSS-treated mice (Fig. 3B) were expected to associate with inflammatory pathways due to the DSS-induced inflammation and resulting immune cell infiltration. The *Apc*$^{Min/+}$ model of tumorigenesis is not inflammation-driven, and differences in immune infiltration and inflammation may be associated with some of the transcriptional changes that distinguish the two adenoma types. The predicted decrease in adhesion of immune cells (Fig. 3B) likely reflects this inflammatory component, while other enriched categories hint at functional decreases in phenotypes relevant to cancer progression, albeit without statistical significance to justify a prediction.

The additional control analysis of 830 transcripts that increased or decreased by at least 2-fold (FDR < 0.1) in both adenoma types (Fig. 3C) yielded results consistent with the predicted activation of canonical Wnt signaling in both types of adenomas. Functions linked to canonical Wnt signaling include proliferation of crypt cells and maintenance of

an undifferentiated progenitor cell fate. The predicted decrease in transport of molecules (particularly ion transport) is consistent with loss of differentiated colorectal epithelium relative to normal colon tissue, as this layer normally functions to absorb water and electrolytes. This proposed loss of differentiation is also consistent with reduced cell migration, a process coupled to differentiation in the colorectal epithelium (26). Interestingly, upstream regulation by the interleukin-10 receptor (IL10RA) was predicted to decrease significantly, indicating that the synthesis of proinflammatory cytokines (blocked by IL10RA) may be high in both adenoma types, likely due to the presence of infiltrating immune cells (27).

Adenomas from *Apc*$^{Min/+}$ mice exhibit unique transcriptional changes consistent with both transcriptional and non-transcriptional tumor suppressor functions of the Apc protein, associating with pathways such as cell proliferation, formation of cellular protrusions and apoptosis (Fig. 3A). IPA predicts increases in cell proliferation and viability and suggests a corresponding decrease in apoptosis as well. Apc is known to control proliferation by antagonizing the transcription of canonical WNT target genes such as those encoding c-Myc and Cyclin D1 (28,29). APC also regulates the formation of membrane protrusions (30,31), interacts with microtubules and facilitate their attachment to kinetochores (9-11) and links the microtubule network to the actin cytoskeleton (32). Apc promotes apoptosis both by negatively regulating the expression of pro-survival canonical WNT signaling targets such as survivin (33) and by directly localizing to the mitochondria and promoting caspase activity (15,34,35). Interestingly, upstream activation of the transforming growth factor-β pathway is predicted, complementing an

opposite effect in the adenomas from AOM/DSS-treated mice. This pathway is tumor suppressive and highly important in the context of colorectal tumorigenesis, but its mutational inactivation in colorectal cancer is typically thought to occur after the acquisition of activated canonical Wnt signaling. The cause of TGFB1 activation and its functional relevance to the gene expression profile of adenomas from $Apc^{Min/+}$ mice is unclear. On the other hand, the expression of transcripts involved in regulation of cell proliferation, the formation of cell protrusions and apoptosis in adenomas from $Apc^{Min/+}$ mice suggests that these pathways may become dysregulated in Apc-deficient adenomas by mechanisms both dependent upon and independent of canonical WNT signaling.

We considered the possibility that Apc may control the abundance of some of these RNAs independently of transcription, especially in the context of cell protrusions where Apc localizes and functions as an RNA-binding protein (31). We examined five transcripts (*Ankrd25*, *Inpp1*, *Kank2*, *Pkp4*, and *Rab13*) regulated by murine Apc through a transcription-independent RNA-binding function localizing these transcripts to cell protrusions, to evaluate whether a subset of transcripts might be regulated by Apc through a transcription-independent mechanism. Four of five transcripts (*Rab13*, *Pkp4*, *Ankrd25* and *Inpp1*) that physically associate with Apc did not show expression differences relative to normal tissues, while the *Kank2* transcript was decreased in expression in AOM/DSS-derived adenomas only. Our data do not rule out the possibility that Apc binds to these or other migration-related transcripts in the colorectal epithelium, but also do not provide strong support for the conclusion that the RNA-binding function of APC is mediating widespread changes in transcript abundance or that comparison to

APC ChIP-seq data has failed to remove them from the screen prior to downstream analyses.

These results motivated our choice to focus subsequent experiments on transcriptional changes unique to adenomas from *Apc*$^{Min/+}$ mice. Three panels of representative transcripts were assembled from the RNA-seq data to summarize differences in gene expression between adenomas from *Apc*$^{Min/+}$ and AOM/DSS-treated mice in the pathways regulating cell proliferation (Fig. 4A), the formation of cell protrusions (Fig. 4B) and apoptosis (Fig. 4C). The canonical WNT signaling target genes *Myc* and *Ccnd1* were included as controls that confirm that the canonical WNT pathway is activated in both types of adenoma. Panel 4A and 4C include *Yap1* and *Fn1*, respectively. Both are known targets of canonical Wnt signaling, indicating that targets of this pathway had not yet been completely removed by the screening process from the list of 231 transcriptional changes found in adenomas from *Apc*$^{Min/+}$ but not AOM/DSS-treated mice.

Transcripts linked to proliferation (Fig. 4A) encode proteins such as growth factors (*Ctgf*) and transcription factors (*Sox11, Elk1*) that drive cell cycle progression (36). The majority are consistent with the predicted increase in cell proliferation, while notable exceptions include increased *Atf3* and decreased *Egf*.

Transcripts encoding proteins linked to apoptotic pathways (Fig. 4B) include a number of reported anti-apoptotic regulators such as *Bdnf*, *Fasn*, *Serpine2* (37), *Msrb3* (38) and *Timp2*; these increased in colon adenomas from *Apc*$^{Min/+}$ mice relative to non-tumor colon tissue. This group also includes transcripts encoding pro-apoptotic regulators such as *Slc20A1*decreased in colon adenomas from *Apc*$^{Min/+}$ mice relative to non-tumor colon

tissue. This trend is consistent with phenotypes of Apc loss (15,39), as the majority of transcriptional changes promote evasion of apoptosis and a smaller number (such as increased expression of *Noa1* (40)) exert a pro-apoptotic effect.

Transcripts linked to chromosome segregation and cytoskeletal organization (Fig. 4C) encode kinesins and other microtubule-associated proteins (*Kif2a*, *Map6* (41)), as well as intermediate filament components (*Vim*) and other proteins involved in cell adhesion and cell motility (*Tpbg*). Some are linked to the regulation of the actin cytoskeleton (*Rhod*, *Mprip* (42)) and cell protrusions in neuronal cells (*Amigo*, *Mprip*, *Ptn*). These findings are consistent with abnormalities in chromosome segregation (10,43) and directional migration (44,45) following Apc loss in the colorectal epithelium.

Filtering transcripts differentially expressed in adenomas from $Apc^{Min/+}$ mice using datasets from mouse models of canonical Wnt signaling inactivation

The lists of transcripts unique to each adenoma type were narrowed down by the removal of those differentially expressed by at least 1.5-fold in the opposite adenoma type relative to non-adenoma tissue. Transcripts unique to adenomas from $Apc^{Min/+}$ mice were reduced in this manner from 231 to 148, while transcripts unique to adenomas from AOM/DSS-treated mice were narrowed from 139 to 92 (Fig. 1). We also sought to facilitate the removal of canonical WNT target genes from transcripts unique to adenomas from $Apc^{Min/+}$ mice. This was performed using two publicly available datasets profiling gene expression following inactivation of the gene encoding β-catenin in the mouse intestine (20) or the gene encoding the canonical Wnt signaling transcription factor TCF4/TCF7L2 in human colon cancer cell lines (21). The first dataset identified

1,697 transcripts up- or down-regulated by at least 1.25-fold following β-catenin inactivation in the mouse intestine, relative to the untreated control. No *p*-value cutoff was applied to these fold-changes due to limited sample number. Overlap with these transcripts enabled the removal of 14 overlapping transcripts from those unique to adenomas from $Apc^{Min/+}$ mice, reducing the number of genes of interest from 148 to 134. The second dataset identified 172 transcripts up-regulated both in adenomas and in human colon cancer cell lines following introduction of a dominant negative TCF4/TCF7L2, enabling the removal of one additional overlapping transcript (the known Wnt target *Yap1*) from those unique to adenomas from $Apc^{Min/+}$ mice, reducing the number of genes of interest to 133 (Fig. 1).

Filtering transcripts differentially expressed in adenomas from $Apc^{Min/+}$ mice using APC ChIP-seq data

Human orthologs were identified for 120 of the 133 genes differentially expressed in colon adenomas from $Apc^{Min/+}$ mice and for 81 of the 92 genes differentially expressed in colon adenomas from AOM/DSS-treated mice. These were compared with an APC ChIP-seq dataset generated from the HCT-116 human colon cancer cell line that is wild-type for *APC* but mutated at *CTNNB1*. APC-associated genomic regions were identified by immunoprecipitation from the chromatin fraction of these cells, followed by massively parallel sequencing (ChIP-seq), as described previously (22). ChIP-seq data identified 44,771 APC-associated genomic regions, of which 12,840 occurred within 10 kb upstream or downstream of a transcription start site, facilitating identification of

candidate target genes. 81 (68%) of the 120 genes differentially expressed in colon adenomas from *Apc*$^{Min/+}$ mice were encoded within 10 kb of an APC-associated genomic region (Fig. 1). APC protein was hypothesized to regulate the expression of some these 81 transcripts through interaction with chromatin. Previous reports show that at least some APC-associated chromatin binding peaks control the transcription of canonical WNT target genes (46,47), while we hypothesize that other peaks control WNT-independent target genes. The latter group of target genes was expected to change in expression only in Apc-deficient adenomas (from *Apc*$^{Min/+}$ mice), while canonical WNT target genes change in expression in adenomas from both *Apc*$^{Min/+}$ and AOM/DSS-treated mice (16).

In silico analysis of gene expression data from human colorectal tumors

The relationship between gene expression and clinical variables in human colorectal tumors was examined using The Cancer Genome Atlas (TCGA) RNA-seq data from patient tumors with known clinical outcomes. This analysis focused on 81 transcripts of interest shown to be differentially expressed in colon adenomas from *Apc*$^{Min/+}$ mice and encoded by genomic loci bound by chromatin-associated APC. Each transcript was considered as a potential marker that might distinguish human colorectal cancer subtypes. Increased expression of the Metastasis-Associated in Colon Cancer 1 (*MACC1*) gene defines higher stage tumors and poor survival subgroups of patients with Stage I, II and III colorectal tumors (24,48), suggesting that the metastatic potential of a colorectal tumor can be defined as early as the onset of invasion and that some markers of

metastasis can be detected within primary tumors. These reports prompted the inclusion of *MACC1* as a critical control in our studies.

Tumors were sorted into early stage (I and II) and late stage (III and IV) tumors, and expression of each gene was compared between the two subgroups. Genes that increased in expression in $Apc^{Min/+}$ adenomas relative to non-adenoma tissue were hypothesized to increase in expression from early to late stage tumors, consistent with the larger hypothesis that these transcriptional changes help drive the progression of APC-deficient adenomas. Eleven such genes increased significantly in expression in Stage III and IV tumors relative to Stage I and II tumors (one-tailed Student's *t*-test, FDR < 0.05), while one such gene (*NOA1*) decreased significantly (FDR < 0.05) instead (Fig. 5). qPCR validation was performed in an independent set of three colon adenomas and three non-adenoma control tissues from each mouse model (Fig. S1), confirming that all 12 transcripts of interest were significantly increased in expression in colon adenomas from $Apc^{Min/+}$ mice relative to their counterparts from AOM/DSS-treated mice. Only *Slc12a4* also exhibited increased expression in the adenomas from AOM/DSS-treated mice compared to the matched control tissues, conforming to the expected pattern of expression for a canonical Wnt target gene (Fig. S1). APC-bound genomic regions identified by ChIP-seq and associated with the loci encoding these transcripts are listed in Figure S2. Following qPCR validation, expression of the twelve transcripts of interest was examined in gene expression data from 242 human colorectal tumors of all stages from the "TCGA" dataset. Tumors were again sorted by stage and expression of each gene was compared in early stage (I and II) vs. late stage (III and IV) tumors (Fig. S3).

Most of the 12 transcripts exhibited a similar pattern of higher expression in late stage tumors, but in most cases without reaching statistical significance. Only the *SERPINE2* transcript exhibited significantly higher expression in late stage tumors (FDR = 0.039), as did the *MACC1* gene (FDR = 0.039), a useful control known to increase in expression in more advanced colorectal tumors (Fig. S3).

Analysis of gene expression as a potential marker for disease-free survival

Clinical data measuring time to recurrence or death was available for the 357 tumors from the "TCGA Provisional" dataset, provided in the "Disease-Free (Months)" category, or in the "Overall Survival (Months)" category when the former was not available. Tumors were sorted into two groups for each transcript of interest, based on expression levels higher or lower than the median. Kaplan-Meier curves (Fig. S4) were constructed comparing disease-free survival in the high-expression and low-expression subgroups, and statistical significance was evaluated using the Cox-Mantel Log-Rank test (FDR < 0.05). High expression of three transcripts (*MAP6*, *PXDC1* and *MSRB3*) was associated with shorter time to recurrence or death, as was high expression of the *MACC1* positive control (Fig. S4). Other transcripts of interest either trended similarly but failed to meet the threshold for statistical significance (such as *MPRIP*, shown) or exhibited little correlation to disease-free survival (such as *SERPINE2*, shown, and others not included in the figure). Validation was attempted through a similar analysis of *MAP6*, *PXDC1* and *MSRB3* expression in 242 tumors from the smaller "TCGA" dataset, but merely trended in the same direction without meeting the required threshold for statistical significance

(Fig. S5). We hypothesize that these findings reflect the shortcomings of the validation dataset rather than of the markers, as median follow-up times for patients in the "TCGA" (validation) and "TCGA Provisional" (discovery) datasets are 0.99 months and 10.07 months, respectively. Accordingly, *MAP6*, *PXDC1* and *MSRB3* merit further study as potential markers as more complete patient outcome data become available. Another large dataset using a microarray platform was available through NCBI's Gene Expression Omnibus (accession number GSE39582, (49)) and similarly showed a relationship between disease-free survival and expression of *MACC1*, *MAP6* and *MSRB3* that matched the expected trends but did not meet the requirement for an FDR < 0.05 (data not shown).

Analysis of gene expression as a potential marker for APC mutational status

The expression of some known canonical WNT target genes correlates well with *APC* status while others do not, suggesting that malignant colorectal tumors may be more complex than colorectal adenomas. Nevertheless, genes increased in expression in $Apc^{Min/+}$ adenomas relative to non-adenomatous tissue were hypothesized to exhibit increased expression in *APC*-mutant tumors relative to their counterparts with wild-type *APC*. Gene expression of *SERPINE2* was examined in 242 tumors from the "TCGA" dataset, of which 203 had been sequenced at the *APC* locus (Fig. 6). Tumors were divided into two subgroups of 55 "*APC* wild-type" tumors (including those with missense mutations) and 148 '*APC* mutant" tumors (including those with frameshift mutations or other premature stop codons detected in either or both alleles of *APC*). *SERPINE2*

expression was predicted to increase in *APC* mutant tumors relative to the wild-type counterparts. The *CCND1* and *MYC* genes were similarly evaluated as controls to illustrate the larger trend that expression of some canonical WNT target genes such as *MYC* correlates with *APC* status in malignant tumors while other targets such as *CCND1* do not exhibit this pattern. The *MACC1* gene was evaluated as a negative control unrelated to *APC* status. Statistical significance was evaluated using one-tailed Student's *t*-test (FDR < 0.05). *SERPINE2* expression is in fact significantly higher in the *APC*-mutant subset than in the *APC*-wild-type group (FDR < 0.0213), albeit to a lesser degree in comparison to *MYC* expression (Fig. 6).

Predictive value of SERPINE2 expression as a potential marker for stage

Positive and negative predictive values (Fig. S6) were calculated to evaluate the ability of high or low *SERPINE2* expression to predict colorectal tumor stage in both the "TCGA Provisional" and "TCGA" datasets, in comparison to *MACC1*. Both datasets indicate that neither *MACC1* nor *SERPINE2* effectively distinguishes early stage (I and II) from late stage (III and IV) colorectal tumors. On the other hand, tumors with low expression of both markers are more likely to be early stage in both datasets. This preliminary result from the intersection of *SERPINE2* and *MACC1* expression data suggest that a panel of such markers may be able to predict tumor stage in a manner that complements existing methods of detecting disease dissemination. This would address a current need in diagnosis whereby undetected microscopic metastatic dissemination to lymph nodes is a

significant driver of adverse outcomes in patients initially diagnosed with “Stage II” disease (50).

Discussion

This study was designed to separate the transcriptional changes that occur following loss of Apc function into two groups: those mediated by its role in canonical WNT signaling (shared between *Apc*-mutant and *Ctnnb1*-mutant adenomas) and those controlled independently of canonical WNT signaling (unique to *Apc*-mutant adenomas). Analysis of shared targets indicates that the strategy was a successful one, as the results are consistent with previous reports that *Apc*-mutant and *Ctnnb1*-mutant adenoma models share widespread transcriptional similarities due to activation of the canonical WNT signaling pathway (16,17). 830 transcripts similarly altered in both types of adenoma relative to their respective non-tumor colon tissue controls (Fig. 1) included many well-known targets of canonical WNT signaling in the colorectal epithelium as well as novel direct and indirect targets of the pathway. The majority of the canonical WNT signaling targets, including *Myc* and *Ccnd1*, exhibit increased expression to similar degrees in both types of adenomas relative to control tissue (Fig. 2).

A smaller group of 231 transcriptional changes was unique to colon adenomas from $Apc^{Min/+}$ mice. These genes constitute a heterogeneous group whose expression is likely controlled by a variety of mechanisms. The identification of *Yap1* and *Fn1* among them demonstrates that the list includes some transcripts controlled by Apc in collaboration with β-catenin and canonical Wnt signaling, but that change more dramatically in

adenomas from *Apc*$^{Min/+}$ mice. Other targets may be associated with pathways of immune infiltration and inflammation, as the degree of immune involvement is one of the factors distinguishing adenomas from *Apc*$^{Min/+}$ mice from their inflammation-driven counterparts (from AOM/DSS-treated mice). Also of great interest are transcripts associated with cell proliferation, formation of cellular protrusions and the regulation of apoptosis that are altered in expression only in *Apc*$^{Min/+}$-derived adenomas (Fig. 3A). These functional pathways are relevant to tumorigenesis and linked to known phenotypes of APC loss (8,11,15,39). We applied several screening steps (Fig. 1) to remove those targets changed in expression due to activation of canonical Wnt signaling or inflammation and to focus on those potentially regulated by chromatin-associated Apc (22). The mechanism by which Apc loss alters their expression is a subject of ongoing investigation. Future studies will identify transcription factor binding sites and protein-protein interactions required for Apc association and will test the hypothesis that chromatin-associated Apc regulates the transcription of these genes in a Wnt-independent manner. At the same time, the enrichment of transcripts involved in the formation of cell protrusions and neuronal migration suggest a possible link to the function of APC as an RNA-binding protein responsible for localizing key target transcripts to areas of dynamic microtubule organization (51). Future studies should also consider the possibility that transcription (and translation) of these genes may also be controlled by Apc independently of the chromatin fraction.

A significant critique raised during the review process is the caveat that some of the gene expression differences between adenomas from *Apc*$^{Min/+}$ and AOM/DSS-treated mice are

due to the inflammatory nature of the AOM/DSS model and the different degrees of immune infiltration into the adenoma types. The AOM/DSS-treated model was chosen because of other advantages, such as the characterized tendency to develop colonic adenomas as opposed to small intestine adenomas, providing a closer match to colon adenomas from the *Apc*$^{Min/+}$ model and a good pair to enable comparison to human colorectal cancers. On the other hand, using an inflammation-based model made it critical to reduce biases, even beyond the screening requirement that the transcripts of interest must change in adenomas from *Apc*$^{Min/+}$ mice relative to adjacent normal tissues in the first step of the screen. Publicly available data from two published studies of β-catenin-driven tumorigenesis in the mouse intestine (20) and human cell lines (21) were used to generate transcript lists that were then used to subtract common transcripts from the list of those changed in adenomas from *Apc*$^{Min/+}$ mice. This led to more complete subtraction of β-catenin-driven targets, previously obscured by inflammation-related noise. Much less evidence of inflammation was observed in gene ontology of transcriptional changes from *Apc*$^{Min/+}$ adenomas following the reviewer recommendations.

Analysis of transcriptional changes from *Apc*$^{Min/+}$ adenomas in the cell proliferation and apoptosis pathways reveals a trend in which many of the genes that increase in expression encode proteins that promote proliferation or inhibit apoptosis. This combined tendency to support proliferation and evasion of apoptosis is consistent with the hypothesis that the greater ability of *APC*-mutant adenomas to progress to malignancy relative to *CTNNB1*-mutant adenomas (5) is partially rooted in differences in gene expression. Among

patients with colorectal tumors, high expression of *SERPINE2* correlates with more advanced stage (Fig. 5). This finding is consistent with known functions of its protein product. *SERPINE2* encodes the Serpin peptidase inhibitor, Clade E (also known as glia-derived nexin or nexin, plasminogen activator inhibitor type 1, member 2), a secreted protein that can inhibit the serine proteases thrombin, urokinase, plasmin and trypsin (52). While gene ontology identified it as an anti-apoptotic molecule (Fig. 4), SERPINE2 interacts with extracellular matrix components such as heparin and heparan sulfate (53); its inhibition of thrombin promotes neurite extension (52). In the context of colorectal cancer cell lines, it exerts a pro-tumorigenic effect by inhibiting a serine protease known as prostasin (54). Its expression is required for anchorage-independent growth, cell migration and xenograft tumor formation in both *APC* wild-type and *APC* mutant colorectal cancer cell models (55). Therefore, despite the strong correlation between *SERPINE2* levels and *APC* status in colorectal cancer, it is likely that overexpression can be triggered independently of *APC* loss, with similar consequences for tumor progression. SERPINE2 also promotes extracellular matrix production and invasion in a pancreatic cancer model (56). These functions are consistent with its increased expression in higher stage tumors (Fig. 5) and with the hypothesis that transcripts aberrantly expressed specifically in *APC*-mutant tumors contribute to tumor progression. *SERPINE2* expression correlates significantly with *APC* status (Fig. 6) but not with disease-free survival (Fig. S4). Rather, its future utility may hinge upon its ability to predict tumor stage (Fig. S6). The ability of *SERPINE2* expression levels to predict colorectal staging is somewhat less than that of the established marker *MACC1*, and

combined use of low *SERPINE2* and low *MACC1* exhibits a modestly enhanced ability to identify a subset of primarily early-stage colorectal tumors. The future utility of *SERPINE2* may hinge upon its ability to predict tumor stage not in isolation, but in the context of a larger panel of multiple markers. It remains to be investigated experimentally whether or not APC binding to the *SERPINE2* promoter in HCT-116 cells constitutes a functional interaction regulating its expression.

Preliminary studies have been added to the Supplementary Materials section (Figures S7 and S8), including characterization of an APC-associated genomic region approximately 47-kb upstream of the *SERPINE2* transcription start site that may be conversely repressed by canonical WNT signaling (Fig. S7). This raises the possibility that *SERPINE2* expression may be controlled by APC in part through canonical WNT signaling. Future experiments will similarly characterize APC effects on a predicted transcriptional element in the proximal *SERPINE2* promoter bound by chromatin-associated APC (Fig. S2). Initial attempts to detect expression changes in colon cancer cell lines wild-type and mutant for *APC* did not detect significant changes in *SERPINE2* following *APC* modulation at the time points investigated (Fig. S8). This is typical of some well-characterized APC-regulated genes such as the canonical WNT target *MYC* (Fig. S8), and may reflect the complexity of cell line models with a baseline of activated canonical WNT signaling as a context in which to study APC-dependent effects. This may be explained in part by the tendency of APC modulation to trigger negative feedback through the canonical pathway.

While *SERPINE2* expression was the sole transcriptional marker identified in adenomas from $Apc^{Min/+}$ mice that significantly correlated with clinical variables in multiple human tumor datasets, *MAP6*, *MSRB3* and *PXDC1* were identified as potential markers of shorter disease-free survival when expressed at high levels (Fig. S4). Like *SERPINE2*, *MAP6* encodes a protein product (microtubule-associated protein 6) best known in neuronal cell types, where it binds and stabilizes microtubules (41) and functions in the formation of neuronal cell protrusions (57). *MSRB3* encodes methionine sulfoxide reductase B3, an enzyme linked to apoptosis that localizes in part to the mitochondria and exerts anti-apoptotic functions (38). *PXDC1* encodes PX domain containing 1, a protein of unknown function that includes a predicted phosphatidylinositol binding domain, possibly enabling it to associate with the plasma membrane and implicating it in membrane trafficking. All three targets exhibit expression patterns significantly associated with stage (Fig. 5) and disease-free survival (Fig. S4) in the larger "TCGA Provisional" dataset, but fall short of the threshold for significance in the smaller "TCGA" dataset (Fig. S5). These genes of interest merit further validation as larger or more complete datasets become available.

These correlations to clinical outcomes in malignant tumors are not sufficient to prove that APC deficiency drives transcriptional changes in adenomas that persist continuously into late stage adenocarcinomas. Changes in the transcription of targets such as *SERPINE2* during colorectal cancer progression cannot be attributed solely to *APC* mutational status, but rather indicate that APC-driven transcriptional changes in adenomas may exert additive effects on apoptosis and cell motility that contribute to

tumor progression and are absent from *CTNNB1* mutant tumors with wild-type *APC*. Future studies in human colon cancer cell lines will be required to characterize the mechanism(s) by which APC regulates each novel transcript involved in apoptosis or cytoskeletal organization, and to determine the extent to which APC effects on their transcription are due to WNT-independent or WNT-dependent mechanisms. Collectively, the identification of these markers may supplement current studies of tumor staging to predict microscopic metastatic dissemination with greater accuracy.

Acknowledgements

Funding was provided by NIH Awards R01CA063507 (J. Groden), UL1TR001070 (J. Groden), and F31CA174260 (W. Hankey), as well as by HHMI MED into GRAD (W. Hankey), and Pelotonia Fellowship Program (K. Ebede, W. Hankey). The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding institutions or the National Institutes of Health.

References

1. Pricolo VE, Finkelstein SD, Wu TT, Keller G, Bakker A, Swalsky PA, et al. Prognostic value of TP53 and K-ras-2 mutational analysis in stage III carcinoma of the colon. American Journal of Surgery 1996;171(1):41-6.
2. Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, et al. American Society of Clinical Oncology provisional clinical opinion: Testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology 2009;27(12):2091-6.
3. Conlin A, Smith G, Carey FA, Wolf CR, Steele RJ. The prognostic significance of K-ras, p53, and APC mutations in colorectal carcinoma. Gut 2005;54(9):1283-6.

4. Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. Cancer Res 1998;58(6):1130-4.
5. Samowitz WS, Powers MD, Spirio LN, Nollet F, van Roy F, Slattery ML. β-Catenin mutations are more frequent in small colorectal adenomas than in larger adenomas and invasive carcinomas. Cancer Research 1999;59(7):1442-4.
6. Faux MC, Ross JL, Meeker C, Johns T, Ji H, Simpson RJ, et al. Restoration of full-length adenomatous polyposis coli (APC) protein in a colon cancer cell line enhances cell adhesion. Journal of Cell Science 2004;117(Pt 3):427-39.
7. Bellis J, Duluc I, Romagnolo B, Perret C, Faux MC, Dujardin D, et al. The tumor suppressor Apc controls planar cell polarities central to gut homeostasis. The Journal of Cell Biology 2012;198(3):331-41.
8. Marshall TW, Lloyd IE, Delalande JM, Nathke I, Rosenblatt J. The tumor suppressor adenomatous polyposis coli controls the direction in which a cell extrudes from an epithelium. Molecular Biology of the Cell 2011;22(21):3962-70.
9. Fodde R, Kuipers J, Rosenberg C, Smits R, Kielman M, Gaspar C, et al. Mutations in the APC tumour suppressor gene cause chromosomal instability. Nature Cell Biology 2001;3(4):433-8.
10. Kaplan KB, Burds AA, Swedlow JR, Bekir SS, Sorger PK, Nathke IS. A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nature Cell Biology 2001;3(4):429-32.
11. Dikovskaya D, Schiffmann D, Newton IP, Oakley A, Kroboth K, Sansom O, et al. Loss of APC induces polyploidy as a result of a combination of defects in mitosis and apoptosis. The Journal of Cell Biology 2007;176(2):183-95.
12. Green RA, Wollman R, Kaplan KB. APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. Molecular Biology of the Cell 2005;16(10):4609-22.
13. Qian J, Sarnaik AA, Bonney TM, Keirsey J, Combs KA, Steigerwald K, et al. The APC tumor suppressor inhibits DNA replication by directly binding to DNA via its carboxyl terminus. Gastroenterology 2008;135(1):152-62.
14. Brocardo MG, Borowiec JA, Henderson BR. Adenomatous polyposis coli protein regulates the cellular response to DNA replication stress. The International Journal of Biochemistry & Cell Biology 2011;43(9):1354-64.
15. Steigerwald K, Behbehani GK, Combs KA, Barton MC, Groden J. The APC tumor suppressor promotes transcription-independent apoptosis in vitro. Molecular Cancer Research : MCR 2005;3(2):78-89.
16. Kaiser S, Park YK, Franklin JL, Halberg RB, Yu M, Jessen WJ, et al. Transcriptional recapitulation and subversion of embryonic colon development by mouse colon tumor models and human colon cancer. Genome Biology 2007;8(7):R131.
17. Reichling T, Goss KH, Carson DJ, Holdcraft RW, Ley-Ebert C, Witte D, et al. Transcriptional profiles of intestinal tumors in Apc(Min) mice are unique from those of embryonic intestine and identify novel gene targets dysregulated in human colorectal tumors. Cancer Research 2005;65(1):166-76.

18. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques 1993;15(3):532-4, 36-7.
19. Kohno H, Suzuki R, Sugie S, Tanaka T. β-catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. Cancer Science 2005;96(2):69-76.
20. Fevr T, Robine S, Louvard D, Huelsken J. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Molecular and Cellular Biology 2007;27(21):7551-9.
21. Van der Flier LG, Sabates-Bellver J, Oving I, Haegebarth A, De Palo M, Anti M, et al. The intestinal Wnt/TCF signature. Gastroenterology 2007;132(2):628-32.
22. Hankey W, Chen Z, Bergman MJ, Fernandez MO, Hancioglu B, Lan X, et al. Chromatin binding profiles of the APC tumor suppressor reveal collaborative regulation of gene expression by canonical WNT signaling, APC and the AP-1 transcription factor. Submitted Manuscript 2017.
23. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Science Signaling 2013;6(269):pl1.
24. Shirahata A, Shinmura K, Kitamura Y, Sakuraba K, Yokomizo K, Goto T, et al. MACC1 as a marker for advanced colorectal carcinoma. Anticancer Research 2010;30(7):2689-92.
25. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Science 2003;94(11):965-73.
26. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. The American Journal of Anatomy 1974;141(4):537-61.
27. Ho AS, Liu Y, Khan TA, Hsu DH, Bazan JF, Moore KW. A receptor for interleukin 10 is related to interferon receptors. Proceedings of the National Academy of Sciences of the United States of America 1993;90(23):11267-71.
28. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281(5382):1509-12.
29. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398(6726):422-6.
30. Iizuka-Kogo A, Shimomura A, Senda T. Colocalization of APC and DLG at the tips of cellular protrusions in cultured epithelial cells and its dependency on cytoskeletons. Histochemistry and Cell Biology 2005;123(1):67-73.
31. Mili S, Moissoglu K, Macara IG. Genome-wide screen reveals APC-associated RNAs enriched in cell protrusions. Nature 2008;453(7191):115-9.
32. Moseley JB, Bartolini F, Okada K, Wen Y, Gundersen GG, Goode BL. Regulated binding of adenomatous polyposis coli protein to actin. The Journal of Biological Chemistry 2007;282(17):12661-8.

33. Zhang T, Otevrel T, Gao Z, Ehrlich SM, Fields JZ, Boman BM. Evidence that APC regulates survivin expression: A possible mechanism contributing to the stem cell origin of colon cancer. Cancer Research 2001;61(24):8664-7.
34. Qian J, Steigerwald K, Combs KA, Barton MC, Groden J. Caspase cleavage of the APC tumor suppressor and release of an amino-terminal domain is required for the transcription-independent function of APC in apoptosis. Oncogene 2007;26(33):4872-6.
35. Qian J, Perchiniak EM, Sun K, Groden J. The mitochondrial protein hTID-1 partners with the caspase-cleaved adenomatous polyposis cell tumor suppressor to facilitate apoptosis. Gastroenterology 2010;138(4):1418-28.
36. Alam KJ, Mo JS, Han SH, Park WC, Kim HS, Yun KJ, et al. MicroRNA 375 regulates proliferation and migration of colon cancer cells by suppressing the CTGF-EGFR signaling pathway. International Journal of Cancer 2017;141(8):1614-29.
37. Rossignol P, Ho-Tin-Noe B, Vranckx R, Bouton MC, Meilhac O, Lijnen HR, et al. Protease nexin-1 inhibits plasminogen activation-induced apoptosis of adherent cells. The Journal of Biological Chemistry 2004;279(11):10346-56.
38. Kwak GH, Kim TH, Kim HY. Down-regulation of MsrB3 induces cancer cell apoptosis through reactive oxygen species production and intrinsic mitochondrial pathway activation. Biochemical and Biophysical Research Communications 2017;483(1):468-74.
39. Morin PJ, Vogelstein B, Kinzler KW. Apoptosis and APC in colorectal tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America 1996;93(15):7950-4.
40. Parihar A, Parihar MS, Chen Z, Ghafourifar P. mAtNOS1 induces apoptosis of human mammary adenocarcinoma cells. Life Sciences 2008;82(21-22):1077-82.
41. Bosc C, Cronk JD, Pirollet F, Watterson DM, Haiech J, Job D, et al. Cloning, expression, and properties of the microtubule-stabilizing protein STOP. Proceedings of the National Academy of Sciences of the United States of America 1996;93(5):2125-30.
42. Surks HK, Richards CT, Mendelsohn ME. Myosin phosphatase-Rho interacting protein. A new member of the myosin phosphatase complex that directly binds RhoA. The Journal of Biological Chemistry 2003;278(51):51484-93.
43. Green RA, Kaplan KB. Chromosome instability in colorectal tumor cells is associated with defects in microtubule plus-end attachments caused by a dominant mutation in APC. The Journal of Cell Biology 2003;163(5):949-61.
44. Wong MH, Hermiston ML, Syder AJ, Gordon JI. Forced expression of the tumor suppressor adenomatosis polyposis coli protein induces disordered cell migration in the intestinal epithelium. Proceedings of the National Academy of Sciences of the United States of America 1996;93(18):9588-93.
45. Mahmoud NN, Boolbol SK, Bilinski RT, Martucci C, Chadburn A, Bertagnolli MM. Apc gene mutation is associated with a dominant-negative effect upon intestinal cell migration. Cancer Research 1997;57(22):5045-50.

46. Choi SH, Estaras C, Moresco JJ, Yates JR, 3rd, Jones KA. α-Catenin interacts with APC to regulate β-catenin proteolysis and transcriptional repression of Wnt target genes. Genes & Development 2013;27(22):2473-88.
47. Sierra J, Yoshida T, Joazeiro CA, Jones KA. The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. Genes & Development 2006;20(5):586-600.
48. Ilm K, Kemmner W, Osterland M, Burock S, Koch G, Herrmann P, et al. High MACC1 expression in combination with mutated KRAS G13 indicates poor survival of colorectal cancer patients. Molecular Cancer 2015;14:38.
49. Marisa L, de Reynies A, Duval A, Selves J, Gaub MP, Vescovo L, et al. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. PLoS Medicine 2013;10(5):e1001453.
50. Liefers GJ, Cleton-Jansen AM, van de Velde CJ, Hermans J, van Krieken JH, Cornelisse CJ, et al. Micrometastases and survival in stage II colorectal cancer. The New England Journal of Medicine 1998;339(4):223-8.
51. Preitner N, Quan J, Nowakowski DW, Hancock ML, Shi J, Tcherkezian J, et al. APC is an RNA-binding protein, and its interactome provides a link to neural development and microtubule assembly. Cell 2014;158(2):368-82.
52. Wagner SL, Geddes JW, Cotman CW, Lau AL, Gurwitz D, Isackson PJ, et al. Protease nexin-1, an antithrombin with neurite outgrowth activity, is reduced in Alzheimer disease. Proceedings of the National Academy of Sciences of the United States of America 1989;86(21):8284-8.
53. Cunningham DD, Wagner SL, Farrell DH. Regulation of protease nexin-1 activity by heparin and heparan sulfate. Advances in Experimental Medicine and Biology 1992;313:297-306.
54. Selzer-Plon J, Bornholdt J, Friis S, Bisgaard HC, Lothe IM, Tveit KM, et al. Expression of prostasin and its inhibitors during colorectal cancer carcinogenesis. BMC Cancer 2009;9:201.
55. Bergeron S, Lemieux E, Durand V, Cagnol S, Carrier JC, Lussier JG, et al. The serine protease inhibitor serpinE2 is a novel target of ERK signaling involved in human colorectal tumorigenesis. Molecular Cancer 2010;9:271.
56. Buchholz M, Biebl A, Neesse A, Wagner M, Iwamura T, Leder G, et al. SERPINE2 (protease nexin I) promotes extracellular matrix production and local invasion of pancreatic tumors in vivo. Cancer Research 2003;63(16):4945-51.
57. Deloulme JC, Gory-Faure S, Mauconduit F, Chauvet S, Jonckheere J, Boulan B, et al. Microtubule-associated protein 6 mediates neuronal connectivity through Semaphorin 3E-dependent signalling for axonal growth. Nature Communications 2015;6:7246.

Figure Legends

Figure 1: Screening steps identify unique transcriptional changes in adenomas from *Apc*$^{Min/+}$ mice relative to their counterparts from AOM/DSS-treated mice.

RNA-seq analysis was used to quantify expression in colon adenomas from *Apc*$^{Min/+}$ mice (left) and AOM/DSS-treated mice (right). Transcripts of interest for each adenoma type changed in expression by at least 2-fold relative to adjacent colon tissue from the same mouse model (one-sample *t*-test, FDR < 0.1) and were differentially expressed from the opposite adenoma type (the Student's *t*-test, FDR < 0.05). Additional screening steps for transcripts from *Apc*$^{Min/+}$ adenomas included the subtraction of those changed in other models of activated Wnt signaling, the active selection of those encoded by a locus with an associated APC ChIP-seq binding region, selection of those correlated to human colorectal tumor stage, and qPCR validation using RNA from a second set of adenomas.

Figure 2: Colon adenomas from AOM/DSS-treated and *Apc*$^{Min/+}$ mice (*C57BL/6J*) exhibit transcriptional activation of canonical WNT target genes.

Twenty target genes of the canonical WNT signaling pathway were examined in RNA-seq data generated from adenomas and non-adenoma tissues from AOM/DSS-treated (red) and *Apc*$^{Min/+}$ (blue) mice. Eighteen transcripts changed in expression by at least two-fold in both adenoma types. *Birc5* and *Ephb3* increased by more than 2-fold in adenomas from *Apc*$^{Min/+}$ mice but by less than 1.5-fold in adenomas from AOM/DSS-treated mice. Expression is shown as a fold-change relative to their respective adjacent colon tissue controls. Error bars depict standard deviation among the three adenoma replicates.

Figure 3: Colon adenomas from *Apc*$^{Min/+}$ mice exhibit unique transcriptional changes related to cell proliferation, apoptosis and formation of cell protrusions.

231 transcripts differentially expressed in adenomas from *Apc*$^{Min/+}$ mice (A), 139 transcripts differentially expressed in adenomas from AOM/DSS-treated mice (B), and 830 transcripts differentially expressed in both adenoma types relative to matched adjacent colon tissue (C) were subjected to gene ontology analysis using Ingenuity Pathway Analysis software. Fold-changes in expression were submitted and functional categories predicted to be increased (red) or decreased (blue) are indicated. Statistically significant predictions of increases or decreases were defined by enrichment scores at least 2 standard deviations from the mean (Z-score of great than 2 or less than -2).

Figure 4: Colon adenomas from *Apc*$^{Min/+}$ mice exhibit unique transcriptional changes related to cell proliferation, apoptosis and the formation of cell protrusions.

Representative transcripts of genes that regulate cell proliferation (A), apoptosis (B) and formation of cell protrusions (C) were selected for adenomas from *Apc*$^{Min/+}$ mice. Each panel contains RNA-seq data from 3 colon adenomas and 1 adjacent colon tissue of each type. Expression is shown in each type of mouse adenoma (in red and blue) as a fold-change relative to their respective non-adenoma colon tissue controls. Error bars depict standard deviation between the three adenomas. The Student's *t*-test was performed to assess statistically significant differences in fold-change between adenomas from *Apc*$^{Min/+}$ and AOM/DSS-treated mice (FDR < 0.05). The *Myc* and *Ccnd1* transcripts (indicated by green arrows) were included as negative controls similarly altered in both adenoma types.

Figure 5: Transcriptional changes unique to adenomas from *Apc*$^{Min/+}$ mice correlate with more advanced stage in human colorectal tumors.

Analysis of gene expression in human tumors was performed for 81 transcripts of interest differentially expressed in colon adenomas from *Apc*$^{Min/+}$ mice and encoded by genomic loci bound by chromatin-associated APC. Expression values for each gene were normalized to the median and the boundaries between expression quartiles were used to construct box-and-whisker plots to compare the range of expression values for early stage (I and II) *vs*. late stage (III and IV) tumors. Genes increased in expression in *Apc*$^{Min/+}$ adenomas relative to non-adenomatous tissue were hypothesized to increase in expression from early stage (red) to late stage (blue) tumors. Eleven such genes (shown above) increased significantly in expression in Stage III and IV tumors relative to Stage I and II tumors (one-tailed Student's *t*-test, FDR < 0.05), while one such gene (*NOA1*) decreased significantly (FDR < 0.05) instead. *MACC1* served as a positive control that shows increased expression in late stage tumors. The black line separating the 2nd and 3rd quartiles of each box plot represents the median.

Figure 6: *SERPINE2* is expressed more highly in human colorectal tumors harboring *APC* mutations relative to their counterparts with wild-type *APC*.

Genes increased in expression in adenomas from *Apc*$^{Min/+}$ mice relative to non-adenoma tissue were hypothesized to increase in expression in *APC* mutant human tumors. *SERPINE2* expression was examined in 242 tumors from the "TCGA" dataset, of which 203 had been sequenced at the *APC* locus. Tumors were divided into two subgroups of 55 "*APC* wild-type tumors" (including those with missense mutations) and 148 '*APC* mutant tumors" (including those with frameshift mutations or other premature stop codons detected in either or both alleles of *APC*). Statistical significance was evaluated using one-tailed Student's *t*-test (FDR < 0.05). The *CCND1* and *MYC* genes were evaluated as a pair of negative and positive controls to illustrate the point that only certain canonical WNT targets correlate in expression with *APC* status. The *MACC1* gene was similarly evaluated as a negative control.

Figure 1: Screening steps identify unique transcriptional changes in adenomas from *Apc*$^{Min/+}$ mice relative to their counterparts from AOM/DSS-treated mice.

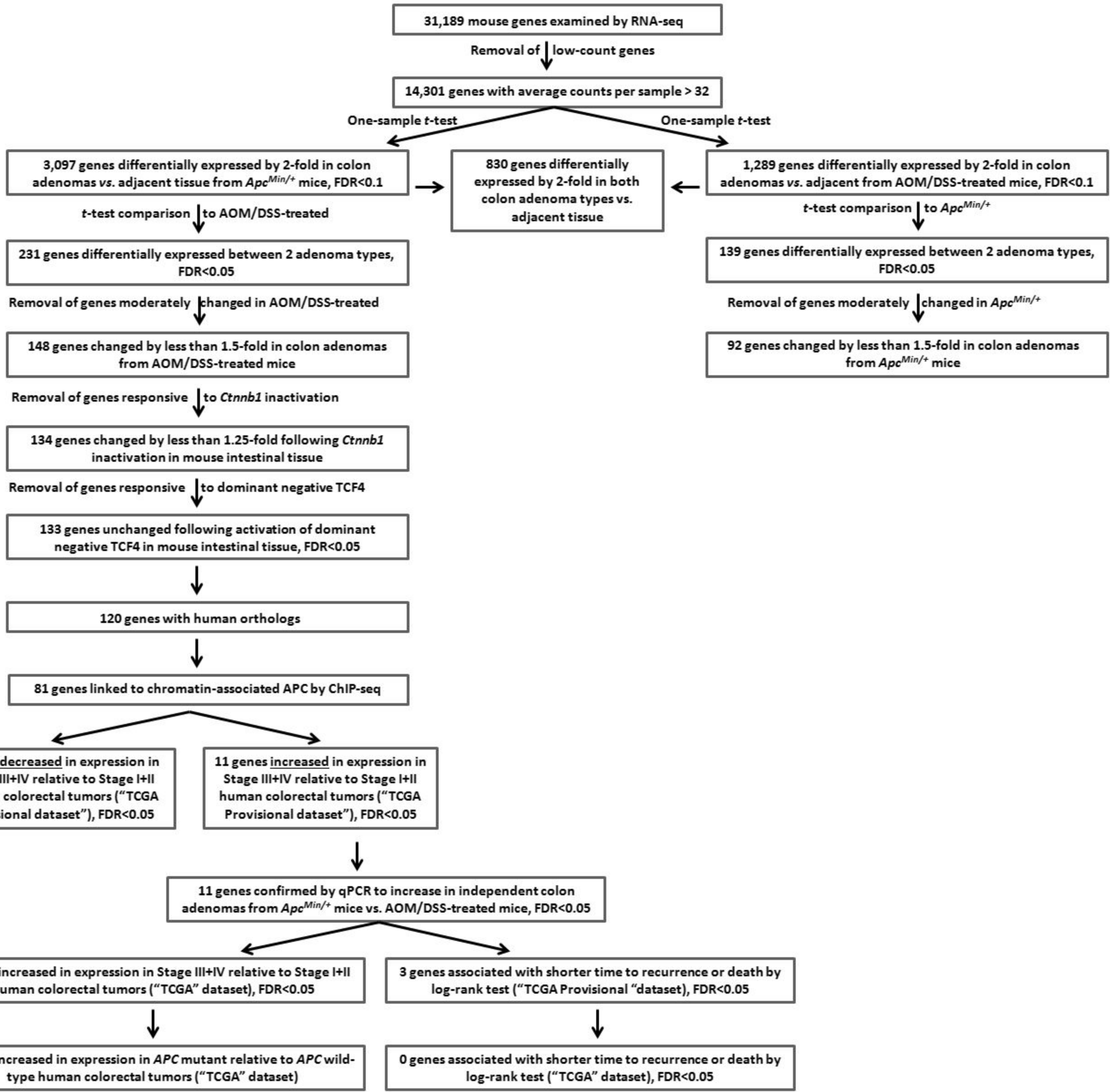


Figure 2: Colon adenomas from AOM/DSS-treated and *Apc*$^{Min/+}$ mice (*C57BL/6J*) exhibit transcriptional activation of canonical WNT target genes.

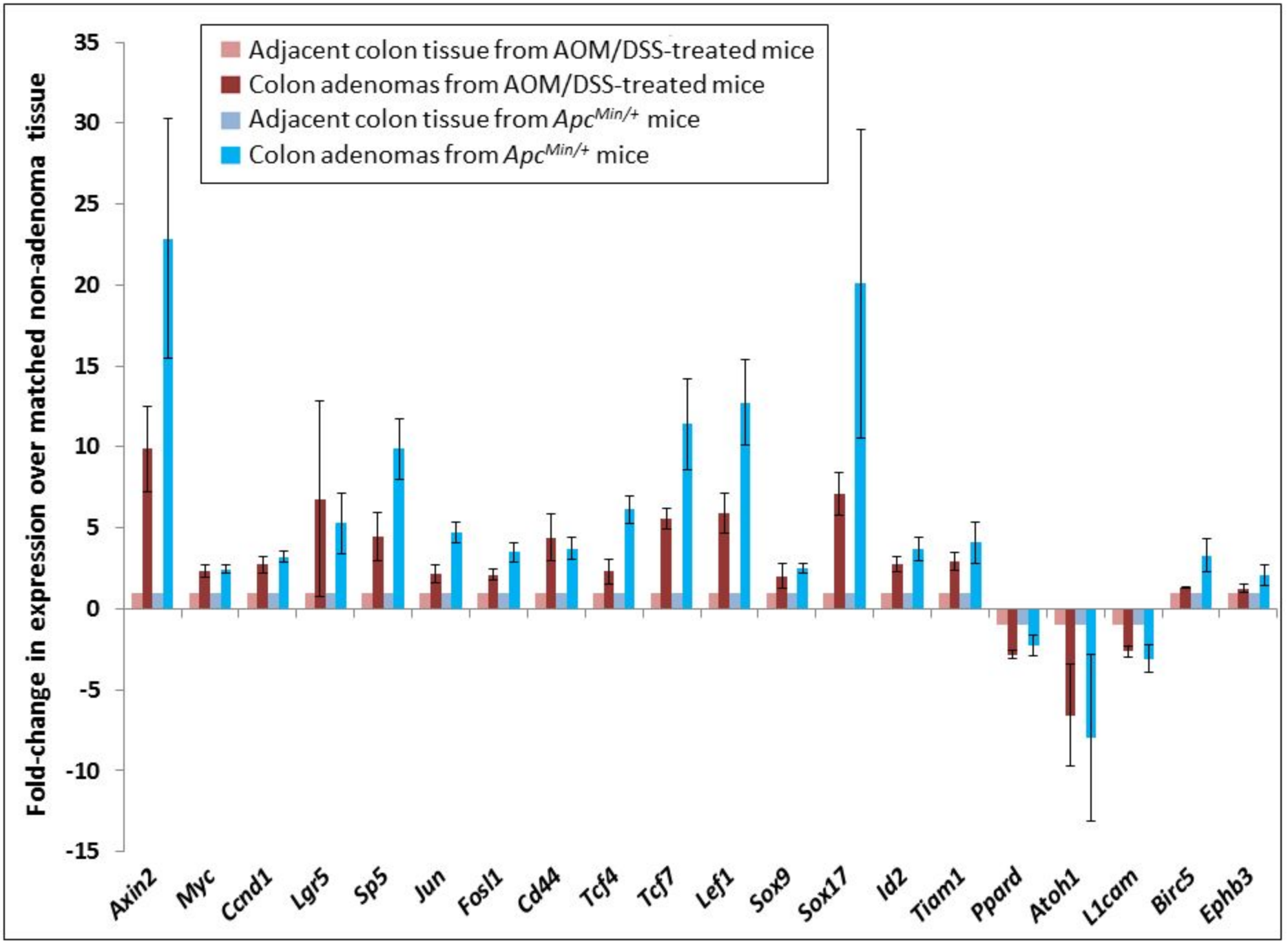
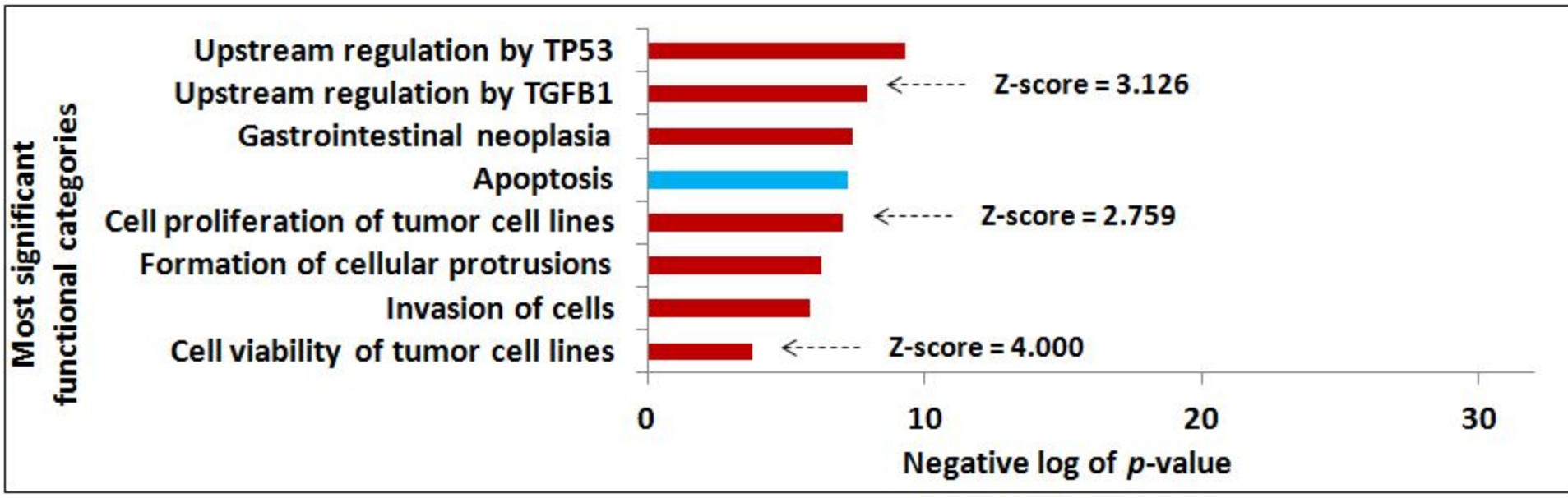
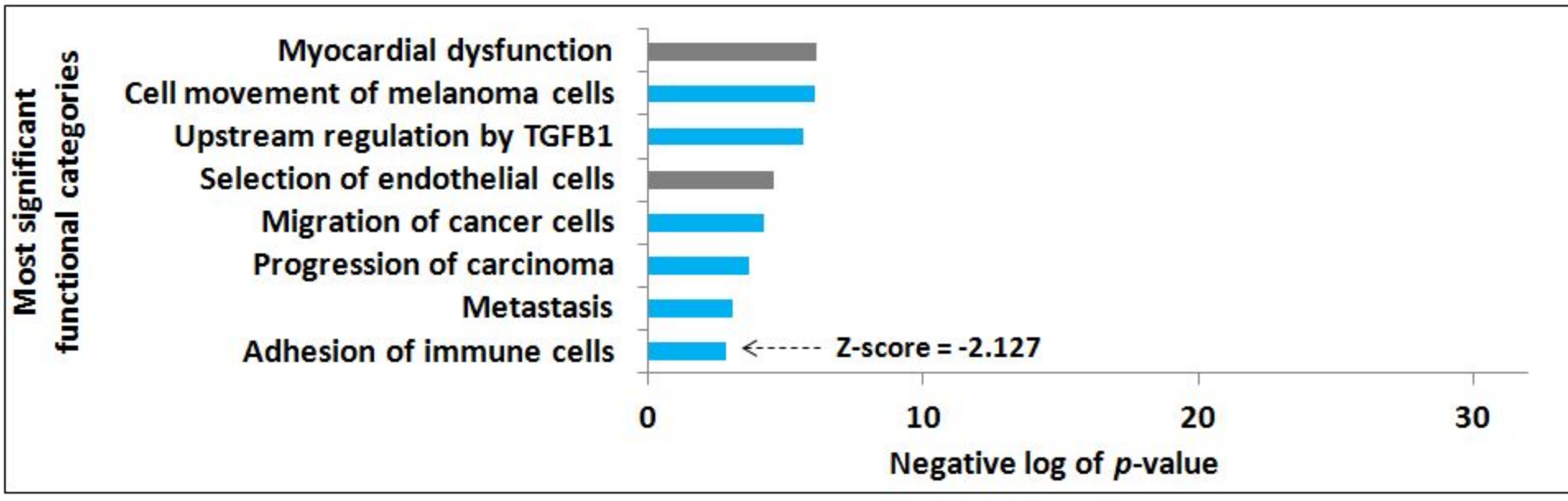


Figure 3: Colon adenomas from *Apc*$^{Min/+}$ mice exhibit unique transcriptional changes related to cell proliferation, apoptosis and formation of cell protrusions.

A.



B.



C.

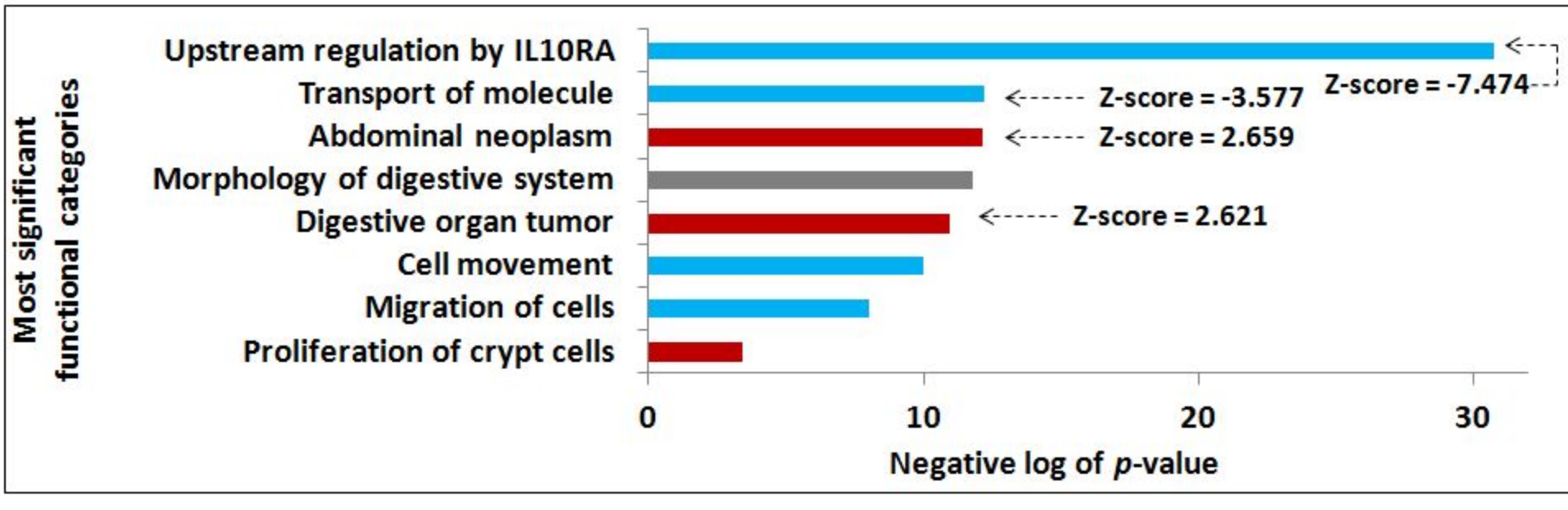


Figure 4: Colon adenomas from *Apc*$^{Min/+}$ mice exhibit unique transcriptional changes related to cell proliferation, apoptosis and the formation of cell protrusions.

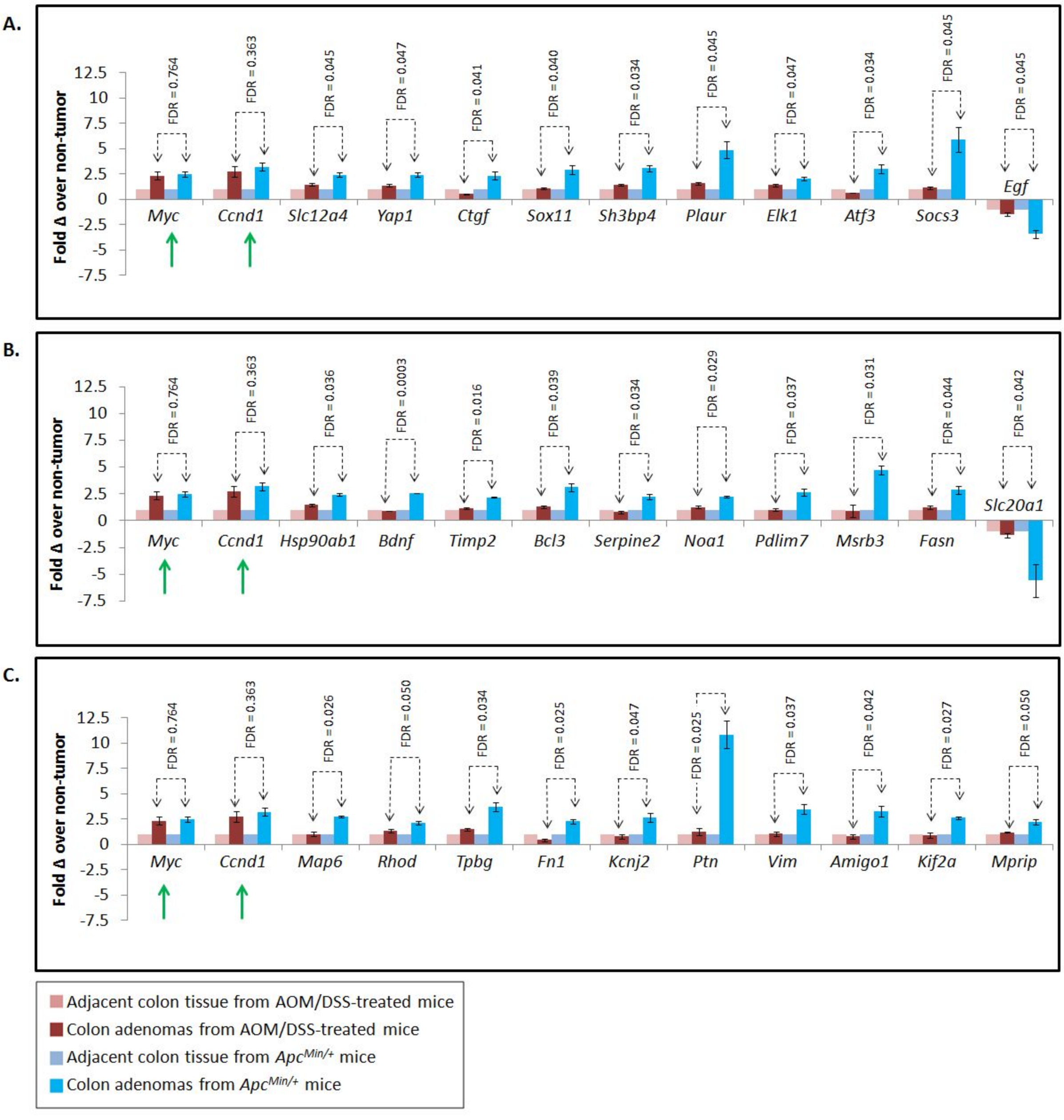


Figure 5: Transcriptional changes unique to adenomas from *Apc*$^{Min/+}$ mice correlate with more advanced stage in human colorectal tumors.

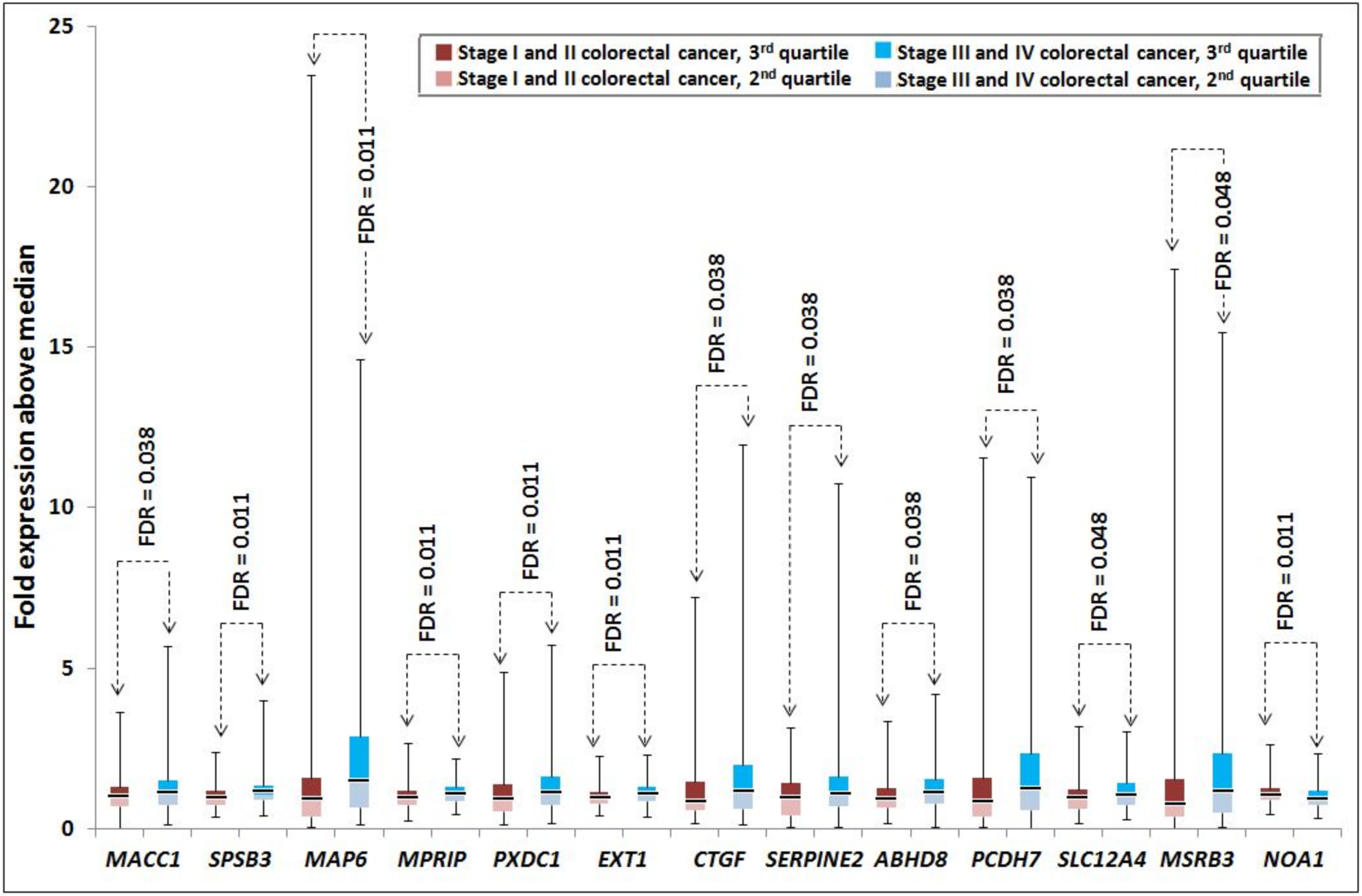


Figure 6: *SERPINE2* is expressed more highly in human colorectal tumors harboring *APC* mutations relative to their counterparts with wild-type *APC*.