

Cell Cycle Control by the APC Tumor Suppressor in Colorectal Cancer

Research Thesis

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by

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ABSTRACT

Colorectal cancer is the second most common cancer that affects both women and men in the United States, with a 50% five year survival if the disease is diagnosed with local metastases (Zeve and Chen, 2009). More than 80% of colorectal cancers are characterized by mutation of the APC tumor suppressor gene, named after the inherited disorder familial adenomatous polyposis coli in which germline APC mutation inevitable leads to colorectal cancer (Grodin et al., 1991). APC functions as a major regulator of the WNT signaling pathway by controlling β -catenin levels in the nucleus and subsequent transcription patterns that promote differentiation (Logan and Nusse, 2004). This work will test the hypothesis that APC has additional tumor suppressor functions independent of β -catenin regulation. Our published in vitro studies have supported this hypothesis by demonstrating APC functions in the inhibition of DNA replication in S-phase and the promotion of apoptosis (Steigerwald et al., 2005; Qian et al., 2007, 2008, 2010). Additionally, APC has been reported to bind to an enhancer site upstream of the *c-MYC* proto-oncogene and to directly downregulate *c-MYC*'s transcriptional activity (Sierra et al., 2006). Consistent with these reports, our preliminary RNA deep sequencing (RNA-seq) data show that a colorectal cancer cell line with activated Wnt signaling exhibits different gene expression profiles when APC is knocked down compared to when APC is expressed normally. A particular interest of this resultant gene profile was a 1.64 fold increase in expression of the CDC25A oncogene when APC was knocked down (Figure 1). CDC25A is a phosphatase that promotes the cell cycle by removing inactivating phosphorylations from the cyclin-- dependent kinases Cdk2 and Cdk4 (Ray et al., 2007). We hypothesize that the loss of APC results in the direct upregulation of CDC25A transcript. The loss of CDC25A regulation

drives the cell cycle to a much more rapid rate similar to that seen in colorectal cancers. The direct binding of APC to specific chromatin could directly influence tumorigenesis and would suggest the need to consider new therapeutic approaches to the treatment of human colorectal tumors without APC.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer related deaths in the Western world and is currently the third most common form of cancer (Zeve and Chen, 2009). In order to develop better diagnostic tests and patient treatments, it is vital to improve our understanding of the pathological progression of this disease. One promising area of research is determining the consequences of mutations in *adenomatous polyposis coli (APC)* gene, which are found in approximately 80% of colorectal cancers (Kinzler *et al.*, 2000). Loss of *APC* leads to the constitutive activation of the Wnt signaling pathway, which controls the transcription of many essential genes in development, cellular proliferation, and differentiation. Loss of this transcriptional regulation by *APC* leads to sporadic polyp formation in the colorectal epithelium (Grodén *et al.*, 1991). If not removed, this pre-cancerous polyp will progress into a malignant tumor. Therefore, it is vital to identify the functions of *APC* that are most important in polyp formation and in the subsequent development of cancer.

APC has long been known to regulate the transcription of cancer related genes indirectly, by targeting the Wnt pathway's key transcriptional regulator β -catenin for degradation (Logan and Nusse, 2004). In addition to that cytoplasmic function, *APC* has recently been

shown to perform nuclear functions that include interaction with chromatin to control transcription directly (Sierra *et al*, 2006). To better understand the consequences of APC mutations in colorectal cancer, it is vital to identify its transcriptional target genes, especially the poorly characterized direct targets. An *in vitro* system was devised to test the direct targets of *APC* exclusively. Whole Transcriptome Shotgun Sequencing was performed on a colorectal cancer cell line with activated Wnt-signaling, either in the presence or absence of APC. Our preliminary findings showed that within our model, APC deficiency results in abnormal activation or suppression of nearly 200 genes. The studies described in this thesis were then focused on validating these findings as well as identifying which individual target genes contribute to cancerous phenotypes. Several targets have been validated and await further functional testing. The goals of these future experiments are to better categorize and develop new therapeutic targets for patients afflicted with APC-deficient colorectal cancer.

PRELIMINARY DATA

Whole Transcriptome Shotgun Sequencing was performed on HCT-116 cells to compare the mRNA profiles of wildtype and APC knockdown cells. A cocktail of 4 siRNAs was used to knock down *APC* mRNA. The

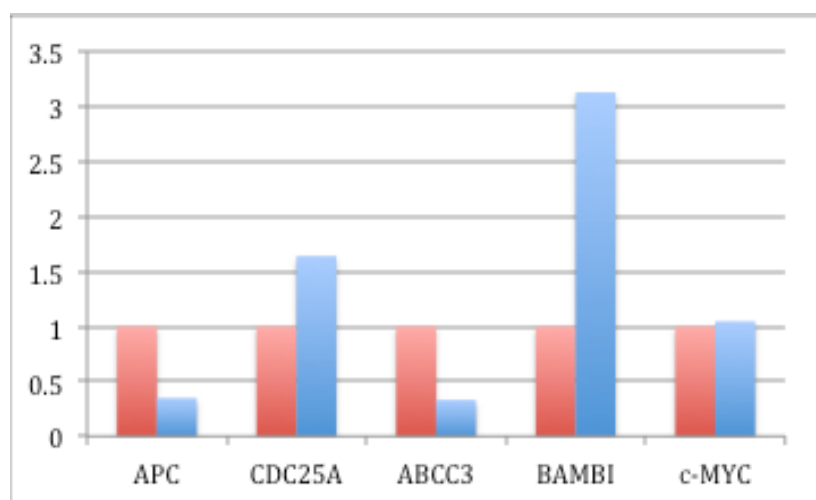


Figure 1
Whole Transcriptome Shotgun Sequence data normalized to fold changes in wildtype APC HCT-116 cells (red) vs. APC deficient APC cancer cells (blue)

mRNA profiles of the cell lines were then measured after 48 hours after the knockdown. The normalized findings (Figure 1) showed a 2.88 fold decrease in *APC*, 1.64 increase in *CDC25A*, 3.03 decrease in *ABCC3*, 3.13 increase in *BAMBI*, and essentially no change in *MYC* mRNA levels. HCT-116 was chosen as a colorectal cancer model based on the cell's continuous activation of Wnt-signaling due to a mutant β -catenin protein that cannot be degraded, despite having wild-type *APC*. This allowed us to identify direct targets of APC specifically because its indirect regulation of gene expression through the Wnt signaling pathway was expected to have a limited impact in a cell line with degradation-resistant β -catenin.

RESULTS

The cell division cycle 25 (CDC25) family of phosphatases provide essential regulation of eukaryotic cell cycles. CDC25A, specifically, is required for progression from G1 to the S phase of the cell cycle. CDC25A is targeted for degradation in response to DNA damage, which prevents chromosomal abnormalities from being replicated and passed on to daughter cells. CDC25A is an identified oncogene, although its exact mechanism in the development of cancer has not been well characterized (Boutros *et al.*, 2007). CDC25A was chosen for further study based on a 1.64 fold increase upon knockdown of APC and its known role in cancer development.

ATP-binding cassette (ABC) proteins transport a variety of molecules across intra- and extra-cellular membranes. ABC subfamily C (ABCC) is specifically involved in excretion of toxins and harmful molecules. ABCC3 is involved in multi-drug resistance but its exact

function and protein interactions have not been determined. ABCC3 could be involved in expulsion of specific chemotherapeutics, leading to further development of tumors. ABCC3 was chosen for further study based on a 3.03 fold decrease upon knockdown of APC and its potential role in cancer therapeutics.

BMP and activin membrane-bound inhibitor (BAMBI) is a transmembrane glycoprotein similar to the type I receptors found in the transforming growth factor-beta (TGF-beta) family. TGF-beta signal transduction is inhibitory of growth and stimulating of differentiation in colorectal epithelium (Massague *et al.*, 2000). BAMBI, however, is a pseudoreceptor for this signaling (Sekiya *et al.*, 2004). BAMBI is thought to limit the ability of TGF-beta to efficiently signal, which limits a cell proliferation and differentiation. BAMBI was chosen for further study based on a 3.13 fold increase upon knockdown of APC and its known interactions in colorectal cancers.

Myelocytomatosis oncogene (MYC) is a nuclear phosphoprotein that is vital to cell cycle progression, cellular transformation, and apoptosis. MYC specifically functions as a transcription factor for many genes involved in the described functions. Overexpression or amplification of MYC has been well characterized and leads to a variety of cancers, including lymphomas in humans (Murry *et al.*, 1983). Of particular interest to this study, MYC is a known target of APC (He *et al.*, 1998). MYC was chosen as a negative control based on a lack of fold change upon loss of APC in our cell models.

Preliminary data from RNA-seq indicate that in APC deficient cell lines, the important tumor transcripts *CDC25A*, *ABCC3*, and *BAMBI* fluctuate to a significant degree. In order to

verify these results, RT-PCR was performed in three technical replicates on the same cDNA that was used for RNA-seq (SYBR Green, Applied Biosystems). Results of this experiment are shown in Figure 2. The RT-PCR results mimic the RNA-seq data very closely. The fold changes for CDC25A, BAMBI, MYC and ABCC3 were 1.73, 2.83, 1.18, and -2.87 respectively.

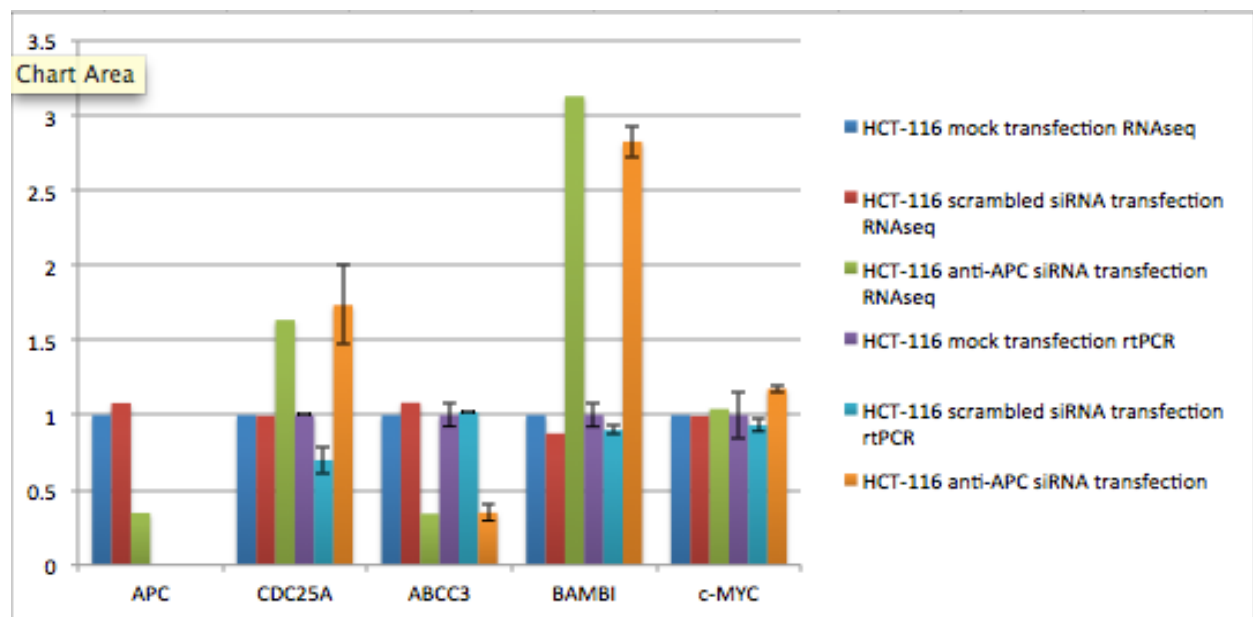


Figure 2

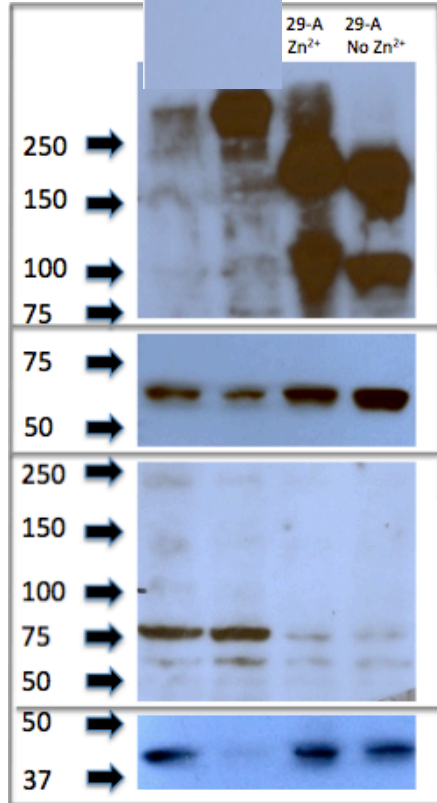
RNA sequencing (blue, red, and green) and real-time Polymerase Chain Reaction (purple, teal, and orange) data shown side by side. Fold changes shown in wildtype APC HCT-116 mock transfected cells were normalized. Scrambled and anti-APC siRNA transfected cell fold changes were then measured against the normalized data.

This represented almost identical fold changes, thus confirming our preliminary data from RNA-seq.

After confirmation of results at the RNA level, Western Blotting was performed in order to confirm results at the protein level. Specific antibodies to APC, CDC25A, ABCC3, BAMBI, and β -actin were obtained from Santa Cruz Biotechnology and were optimized for ideal signal to background ratio. HCT-116 cells were double-transfected on consecutive days with scrambled siRNA and α -APC siRNA. HCT-116 cells were chosen based on the genotype of wildtype APC with constitutively activated β -catenin. Additionally, HT-29-APC cells were

induced for APC expression by addition of Zn²⁺. HT-29-APC cells were chosen based on their ability to reverse the loss of endogenous APC alleles using a transgenic APC under the control of a metallothionein promoter. HT-29-APC provides a reliable complement to hct-

116 based on the ability to control APC expression. Utilizing both cell lines for analysis when APC is present and absent. Therefore when the APC regulated genes are up-regulated in



Primary Antibody	Expected Band
α-APC	Full Length APC 312 kDa
	Truncated APC 200 kDa 100 kDa
α-CDC25A	59 kDa
α-ABCC3	169, 127, 65, 55, and 32 kDa
α-β-actin	42 kDa

one cell line, they should theoretically be suppressed in the other. Both cell lines

Figure 3
Western blot of APC, CDC25A, ABCC3, and β-actin. 50mg of protein per lane and run on a 4-12% gradient gel. 116 = HCT-116 cell line, 29-A = HT-29-APC cell line, α-APC= α-APC siRNA transfection, SCR = scrambled siRNA transfection, Zn²⁺ = zinc induced cells, and No Zn²⁺ = zinc free cells. Antibodies and expected band position listed on right.

were then harvested and whole-cell lysates were obtained. Bradford Assay was then performed in order to determine protein concentration. Proteins were separated by gel electrophoresis on a gradient gel. Proteins were transferred to a polyvinyl difluoride membrane and western blotting was performed.

Upon observation of the western blot for APC, a band of approximately 315 kDa was seen in the HCT-116 protein lanes, with the band being much darker in the scrambled siRNA. In the HT-29-APC cellular protein lanes, bands of approximately 315, 200, and 100 kDa were observed in the zinc induced lanes, but only bands of 200 and 100 kDa were seen in the zinc free cellular protein lanes. The western blot for APC demonstrated the expected band sizes of 312 kDa for full length APC and the appropriate 100 and 200 kDa lengths for truncated APC. This demonstrated that the α -APC siRNA transfection was successful for knocking down APC in HCT-116 cells. The lack of a band at 312 kDa in the HT-29-APC zinc free lane showed that the zinc specific metallothionein promoter was successfully repressed when zinc was absent. A band at 312 kDa in the zinc-induced lane demonstrated the activation of the metallothionein promoter when zinc is present.

The CDC25A western blot demonstrated bands at approximately 60 kDa in all lanes. The bands looked larger in both the α -APC siRNA and zinc free lanes when compared to the scrambled siRNA and zinc induced lanes respectively. Bands at 60 kDa matches the expected CDC25A molecular weight of 59 kDa. Upon further observation, the bands in the scrambled siRNA qualitatively appear to be about half the intensity of the α -APC siRNA bands. Additionally, the zinc free HT-29-APC band also appears about half the strength of the zinc induced. This confirms the RNA sequencing data and rtPCR data, that when APC is downregulated CDC25A is upregulated. This confirmation lead to further investigation of APC and CDC25A influence on cell cycle.

The ABCC3 western showed bands of approximately 250, 140, 110, 80, 65, and 50 kDa in the HCT-116 cellular protein lanes. The bands looked qualitatively the same in each lane.

HT-29-APC cell lines showed bands of approximately 80, 65, and 50 kDa. Again, the bands look approximately the same darkness in both lanes. The expected molecular weights for ABCC3 isoforms recognized by our antibody were 169, 127, 65, 55 and 32 kDa. The only band to match up was the 65 kDa band and it was fairly weak in all four lanes. The strongest band appeared at approximately 80 kDa, which leads to the thought that this is perhaps non-specific binding of other proteins. The lack of relevant bands leads to inconclusive results for ABCC3. Further investigation into cellular concentrations of ABCC3 in our cell lines need to be performed before a conclusion can be drawn about the antibody's specificity.

The β -actin western showed bands of approximately 45 kDa in all lanes. In the HCT-116 lanes, the band is much darker in the α -APC lane and very faint in the scrambled lane. The bands look approximately the same in the HT-29-APC lanes. The only band observed in the β -actin western was at approximately 45 kDa. This observation correlates well with the expected band length of 42 kDa. In the HT-29-APC lanes, it appears that the loading was very similar. However, in the HCT-116 lanes there appears to be a discrepancy in that the α -APC lane appears to be overloaded. This is not a large concern because APC was still successfully knocked down in the overloaded lane. It is a concern regarding the CDC25A interpretation because the underloaded lane showed less CDC25A. However, our preliminary data shows that β -actin shows a 1.26 fold mRNA increase with loss of APC, which could explain some of the discrepancies shown between knockdown and wildtype APC. β -actin upregulation is repeatedly seen in APC knockdown cell lines at the protein level as well.

Based on the results of CDC25A RT-PCR and western blotting, further investigation of APC-CDC25A impact on cell cycle was performed. HCT-116 cells were prepared again with a mock, scrambled siRNA, and α -APC siRNA transfection. HT-29-APC cells were also prepared in an APC induced and uninduced medium. 1.37×10^5 cells of each of the 5 samples were fixed in ethanol and resuspended in a propidium iodide staining solution. The cells were then taken to the Flow Cytometry Lab at The Ohio State University and cell cycle analysis was performed.

Results of HCT-116 cell cycle analysis (Figure 4) showed that in the mock transfection cells,

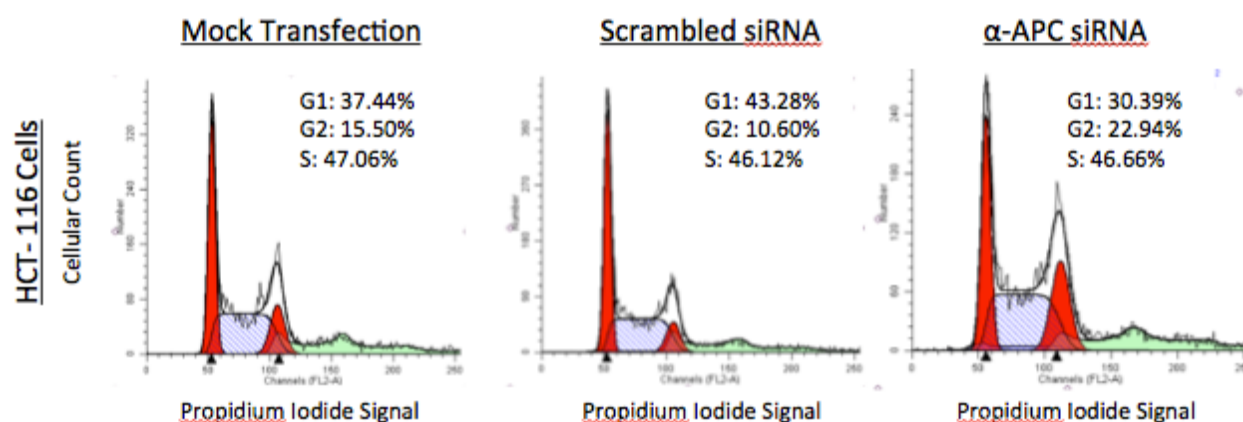


Figure 4
Cell cycle analysis using propidium iodide staining on HCT-116 cells under mock, scrambled siRNA, and α -APC siRNA transfections. G1, S, and G2/M phase percentages were estimated using ModFIT software.

37.44% were in G1, 15.50% were in G2/M, and 47.06% were in S. For the scrambled siRNA transfected cells, 43.28% were in G1, 10.60% were in G2/M, and 46.12% were in S. Lastly, for the α -APC siRNA transfected cells, 30.39% were in G1, 22.94% in G2, and 46.66% in S. The results for HCT-116 cells indicate that G1 to S phase transition increases by 7.05% and 12.89% when an α -APC siRNA transfection is compared to mock and scrambled siRNA transfections respectively. This is an expected result because APC is known to have a role

in suppressing proliferation (Morin *et al.*, 1996). In addition, this follows our hypothesis because when APC is knocked down CDC25A is upregulated and the cell should progress through the G1/S phase checkpoint more efficiently.

Results of HT-29-APC cell cycle analysis showed that in zinc free media cells, 41.92% were in G1, 16.18% were in G2/M, and 41.90% were in S. For the APC induced cells, 53.80% were in G1, 14.47% were in G2/M, and 31.73% were in S.

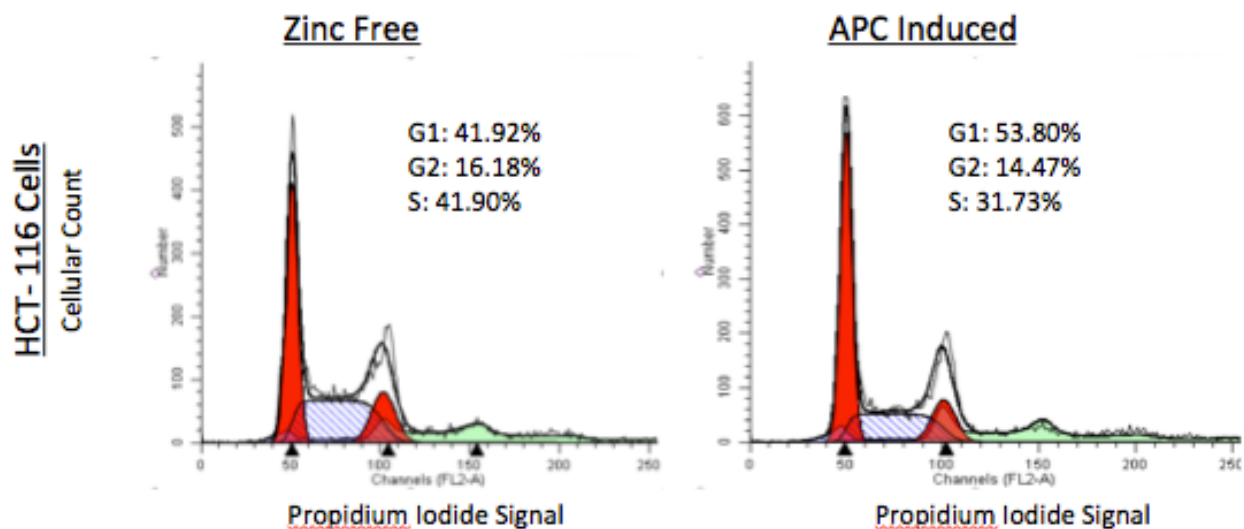


Figure 5
Cell cycle analysis using propidium iodide staining on HT-29-APC cells in zinc free or 100 μ M zinc media. G1, S, and G2/M phase percentages were estimated using ModFIT software.

Again, these results are expected because APC is known to play a role in the downregulation of cell proliferation. However, these results are also consistent with our hypothesis that when APC is present, CDC25A is downregulated and cells should stop at the G1 to S checkpoint more frequently. Therefore when APC is knocked down or absent, as in the α -APC siRNA transfection or uninduced HT-29-APC, the cells should progress more rapidly through the G1 to S phase checkpoint. The results for the HT-29-APC indicate an 11.88% difference when comparing the G1 phase percentages for zinc free vs zinc

induction media. We hypothesize that when APC is knocked down, cellular concentration of CDC25A are elevated and cells spend less time in the G1 phase when compared to wildtype APC. These experiments have established a correlation and the current system allows for further experiments to test causation by CDC25A.

DISCUSSION

There are only two well-characterized targets of APC that are individually sufficient to contribute to tumorigenesis, MYC and cyclin D1 (Shtutman *et al*, 1999; He *et al*, 1998). The purpose of this experiment is to identify other proteins that have well characterized functions and are also potential targets of APC. Once these proteins have been identified, experimental procedures could then be used to determine the significance of the APC-protein interaction. The results of these experiments would potentially identify new therapeutic targets and contribute to the knowledge of tumorigenesis and cancer development.

Whole Transcriptome Shotgun Sequencing was performed on HCT-116 cells to compare the mRNA profiles of wildtype and APC knockdown cells. The results of the RNAseq were used to identify potential targets for APC. Targets were narrowed down and selected based on fold changes and the target's known cancerous properties. In order to confirm transcription levels in the RNA-seq data, RT-PCR was performed on HCT-116 cells using the same cDNAs previously used in the RNA sequencing. 4 targets were confirmed by the RT-PCR experiment. Western blotting was then performed on the 3 targets (CDC25A,

ABCC3, and BAMBI) in order to confirm protein concentration levels in relation to the transcription fold changes shown in the RNA-seq and RT-PCR data.

Western blotting qualitatively confirmed CDC25A protein levels but provided inconclusive results for ABCC3 (Figure 3). ABCC3 still has potential to advance into further experimentation, but a more defined western blot must first be obtained. MYC was not chosen for further study based on a lack of fold changes in the absence of APC. BAMBI had no acceptable antibody, so experimentation was placed on hold until a suitable one could be found.

CDC25A was then chosen for further analysis based on the fact that the western blot results confirmed the experimental hypothesis. Cell cycle analysis by flow cytometry was chosen as a pilot study for the effect of APC changes on the cell cycle. The study was performed using propidium iodide on HCT-116 and HT-29-APC cells. APC cellular concentrations were manipulated and the cells were taken to the Flow Cytometry Lab at The Ohio State University. Cell cycle analysis demonstrated that APC deficient cells progress through the G1/S phase checkpoint at an increase of 7.05% and 12.89% in HCT-116 cells and 11.88% in HT-29-APC when compared to cells with wildtype APC.

CDC25A is a phosphatase responsible for cellular progression through the G1 to S phase checkpoint. This function is pivotal for cellular development and proliferation. As a result of this function, CDC25A has been found to be overexpressed in a variety of cancer, which include breast and colorectal (Sansom *et al.*, 2007; Tetso *et al.*, 1999). Therefore further analysis of APC-CDC25A interaction could provide vital information into how colorectal cancers develop rapidly proliferating phenotypes.

Further analysis of CDC25A must be performed in order to better characterize the relationship between APC and CDC25A, however the pilot study provided a good foundation for further investigation into our hypothesis. In order to test if CDC25A is sufficient to cause progression through the G1/S phase checkpoint when APC is wild type, an overexpression experiment must be performed. This would be accomplished through the use of a vector to transfect CDC25A directly into HCT-116 cells. HCT-116 cells would be used based on the fact that it has wild type APC and lower cellular concentrations of CDC25A. Therefore, overexpression is meaningful. CDC25A overexpression would need to be optimized in order to determine the fold change impact of CDC25A increase.

In order to determine if CDC25A is necessary for cell cycle progression through the G1/S phase checkpoint in cells with loss of APC, a knockdown of CDC25A would need to be performed. This could be accomplished with a α -CDC25A and α -APC siRNA double transfection into HCT-116 cells. Knockdown of both APC and CDC25A is necessary because CDC25A is present at a low cellular concentration in HCT-116 cells. This would model a cancerous human cell in which APC is deficient. This would allow for the characterization of CDC25A's necessity for polyp formation. Again, optimization of the knockdown would be ideal in order to determine the effect of fold decrease of CDC25A. Cell cycle analysis would be performed in both knockdown and upregulated experiments and the results would be compared to controls.

An additional requirement to the knockdown and upregulation experiments would be to perform both experiments in a cell cycle synchronization environment. Serum starvation

can stop cell cycle progression, leaving cells arrested in the G1 phase. Once all cells are in G1 phase, serum could be added back and growth would be allowed to progress. Periodic measurements could then be taken in order to determine if knockdown APC and wildtype CDC25A progress through the G1/S phase checkpoint faster than APC/ CDC25A knockdown cells.

In addition to the short-term future of this experiment, larger goals would be to determine CDC25A's role in cancer *in vivo*, with a focus on determining if CDC25A is necessary for clinical phenotypes observed in colorectal cancers. This could be accomplished through the use of mouse models. A CDC25A deficient transgenic mouse line could be crossed to APC deficient mouse lines, compared to APC deficient mouse lines with wildtype CDC25A. Polyp formation would be allowed to progress and tumor numbers would then be counted. According to the data of this experiment, it would be expected that the CDC25A deficient mouse lines would develop fewer polyps when compared to wildtype CDC25A.

This experiment has provided a hypothesis that in APC deficient cells, CDC25A is overexpressed and cells progress through the G1/S phase checkpoint more rapidly. This provides a foundation for further investigation into APC-CDC25A interaction, with the hopes of characterizing another target gene for APC. If characterized, it could provide another method to target APC deficient cancers. CDC25A is already the target for therapeutics such as quinones (Garuti et al, 2008), which reflects its importance in cancer cells is already well known. CDC25A could become a more important therapeutic target, while also allowing for better diagnosis and prognosis for APC deficient cancer patients.

MATERIAL AND METHODS

APC knockdown in HCT-116 Cells

[The following protocol was generated from the Thermo Scientific DharmaFECT Transfection Reagent Protocol for siRNAs] Scrambled siRNA (not actually a scrambled version of any of the α -APC sequences) and α -APC siRNA pools from Dharmacon were resuspended to a final concentration of 10 μ M in Thermo's 1X siRNA buffer (6mM HEPES pH 7.3-7.6, 60mM KCL, 0.02mM MgCl₂). In 6-well plates, 1x10⁵ HCT-116 cells were plated per well 20 hours before transfection. The HCT-116 cells were cultured in McCoy's 5A media + 10% FBS.

On the day of the transfection 5 μ L of 10 μ M siRNA (scrambled control or α -APC pool) was combined with 195 μ L serum-free McCoy's 5A media and mixed. In a separate tube, 4 μ L DharmaFECT2 transfection reagent was combined with 196 μ L serum-free media and mixed. The tubes were incubated separately at room temperature for 5 minutes, and then combined into a single tube. The combination was incubated for exactly 20 minutes. Along with the scrambled control and α -APC, a mock transfection was set up that contained no siRNA. The transfection mixture was added to 1.6 mL of 10% FBS in McCoy's 5A media.

The media from the HCT-116 well plates was aspirated and replaced with 2mL of the transfection media mixture. The cells were incubated in the transfection mixture for 6 hours. The media was then aspirated and replaced with 2mL of pre-warmed McCoy's 5A media + 10% FBS. The cells were incubated for another 8 hours and an additional media change was performed. After 24 hours, the transfection was repeated exactly as the

previous steps described. The transfection media was again changed 6 hours after the transfection with 2mL of pre-warmed McCoy's 5A media + 10% FBS and again 8 hours later.

In addition to these two media changes, additional media changes were performed 24 and 32 hours after the second transfection, for a total of 4 media changes after the second transfection. The purpose of these changes is to remove residual inactive transfection complexes and transfection reagents, as these will cause cell harm and death. Cells were harvested 48 hours after the second transfection using RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 alternative, 1 mM EDTA, 1 mM PMSF, 1% Roche Mammalian protease inhibitor cocktail) in order to recover whole cell lysates.

APC induction in HT-29-APC cells

HT-29-APC cells were maintained in McCoy's 5A media supplemented with 10% FBS and 0.6 µg/mL Hygromycin B. The HT-29-APC cells were plated in a 6-well plate at a density of 2×10^5 cells/well. 24 hours later, zinc-containing media was prepared to a final concentration of 100 mM. The media was aspirated off of the cells in the 6-well plate and 3 mL of the zinc-chloride media was added to 3 of the wells (the other 3 were left for controls).

The cells were incubated 48 hours before harvesting. APC expression will be higher at 24 hours after zinc-induction than at 48 hours, but the extra day allows more time for APC-driven transcription changes to translate to the protein level of expression. The cells were

harvested in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 alternative, 1 mM EDTA, 1 mM PMSF, 1% Roche Mammalian protease inhibitor cocktail) in order to recover whole cell lysates.

Real Time-PCR

HCT-116 cells were plated and treated with a mock, scrambled siRNA, and α -APC siRNA transfections according to the protocol described above. Total RNA was harvested from each cell condition and a TRUseq RNA-seq sample preparation kit was used to isolate mRNA and prepare cDNA libraries. These samples had previously been analyzed by RNA-seq and the same cDNA was used as a template for this RT-PCR. To set up the RT-PCR, three reactions for each transfection condition was used in a combination of cDNA + protein specific primer. Primers were designed to the coding sequence of BAMBI, ABCC3, CDC25A, and MYC, and were previously optimized for final concentration. Three reactions were set up for each transfection condition in relation to BAMBI according to the follow formula: 1 ng of cDNA and 300 nM of BAMBI primer set. Similarly, for ABCC3: 1 ng of cDNA and 300 nM of ABCC3 primer set were used. A similar formula was used for CDC25A: 1 ng of cDNA and 900 nM of CDC25A primer set. Lastly, for MYC: 1 ng of cDNA and 50 nM of MYC primer set were used. The reactions were brought up to a volume of 10 μ L with nuclease free water and 2X SYBR Green PCR master mix was added in equal volumes. The reactions were then taken to the Shared Resources at The Ohio State University for RT-PCR analysis.

Western Blotting: Preparing Whole Cell Lysates

HT-29-APC and HCT-116 cells were transfected according to the procedure described above. Adherent cells were harvested from 10-cm² plates by washing once with Dulbecco's

PBS and then scraped for removal. The cells were placed in a 15mL Falcon tube and spun for 5 minutes at 1000 rpm (170g). The supernatant was aspirated and the cells were placed on ice. The solid cell volume was estimated to be 30 μ L and 4 volumes (120 μ L) of cold NP-40 lysis buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40 alternative, 5mM EDTA, 1mM PMSF, 1% Roche mammalian protease inhibitor cocktail) was used for APC protein recovery and RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 alternative, 1 mM EDTA, 1 mM PMSF, 1% Roche Mammalian protease inhibitor cocktail) for recovery of whole cell lysates.

The cell pellet was resuspended and transferred to a pre-chilled Eppendorf tube. The mixture was incubated on ice for 15 minutes and then centrifuged at 13,000 rpm for 15 minutes. The supernatant was divided into 30 μ L aliquots and snap-froze by dry ice and ethanol. The samples not in use were stored at -80°C.

Bradford Assay

A series of dilutions of 0 μ g/mL, 5 μ g/mL, 10 μ g/mL, 15 μ g/mL, and 20 μ g/mL was prepared of Bovine Serum Albumin (BSA), and the total volume was brought up to 798 μ L with double-distilled water. 2 μ L of lysis buffer was then added to each BSA control. For each experimental cell sample, 798 μ L of double-distilled water was placed into cuvettes, along with 2 μ L of cellular sample. Into both the control and the experimental samples, 200 μ L of BioRad Protein Assay Dye Reagent (Coomassie Brilliant Blue G-250 Solution) was added into each cuvette. The mixtures were incubated for 10 minutes at room temperature. The measurements for each of the BSA solutions were measured against a the 0 μ g/mL control in order to create a known protein concentration curve. The experimental samples were

then measured against the 0µg/mL control. The protein readings were obtained through the use of a spectrophotometer. The samples were then compared to the standard curve established protein control of BSA in order to determine sample protein concentration.

Western Blotting: Gel Electrophoresis

A precast 4-12% gradient gel (Novex) was loaded with 50µg of protein sample according to the Chart 1 and run for approximately 2 hours at 100 V.

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
Sample	Protein Ladder	α-APC siRNA HCT-116	Scrambed siRNA HCT-116	Zinc Induced HT-29-APC	Zinc Free HT-29-APC

Chart 1

Lane loading sequence for APC, CDC25A, ABCC3, and β-actin western blots

5X transfer buffer (387 mM glycine, 50 mM Tris Base, 5% methanol) was made and the gel was equilibrated with the buffer for 1 minute. The gel was then set up for electrophoresis to a polyvinyl difluoride membrane (PVDF). Electrophoresis was performed for 16 hours at 200 mA at a temperature of 4°C. The PVDF membrane was blocked for 2 hours in TBST that contains both 10% milk.

The primary antibodies for APC, CDC25A, ABCC3, and β-actin were obtained from Santa Cruz Biotechnology. The primary antibodies were placed in 10% milk according to the following concentrations: 1:500 for CDC25A, 1:500 for ABCC3, 1.5 µg/mL for APC, and 1:2500 for β-actin. The membranes were incubated for 18 hours in the primary antibody solution on a shaker at 4°C. After incubation, the membranes were washed with 25 mL of TBST 8 times with solution changes occurring every 5 minutes. The membranes were then incubated in the secondary antibody of corresponding species-specific horseradish

peroxidase (HRP) at a concentration of 1:5000. B-actin antibody has HRP already bound, so the secondary step was not performed. The membranes were incubated in the secondary antibodies for 90 minutes at room temperature. The blots were washed with 25 mL of TBST 14 times with solution changes occurring every 10 minutes.

After washing the membrane, 1 ml each of Western Lightning Enhanced ECL Luminal Reagent and Western Lightning Enhanced ECL Oxidizing Reagent were mixed together. The 2 mL solution was prepared and added to each membrane separately. The membranes were incubated in the solution for 1 minute, placed in plastic wrap, and taken to a dark room for film exposure. The blots were exposed for 30 seconds, 2 minutes, and 30 minutes and the film was developed.

Cell Cycle Analysis by DNA Content

HCT-116 were transfected and HT-29-APC were induced to APC expression according to the protocols described above. The rest of the protocol was performed on ice. The cells were trypsinized and washed in Dulbecco's phosphate buffered saline (PBS). They were then centrifuges for 5 minutes at 200 g at 4°C. They were resuspended again and 2×10^6 cells/mL were placed in new tubes. The cells were centrifuged for 5 minutes at 200 g and 4°C. The PBS wash was removed and the cells were resuspended in 1 mL PBS. The cells were vortexed gently for 30 seconds to break up any cellular clumps. The vortexing was continued on the cell suspension and 7 mL of 80% ethanol (previously chilled to -20°C) was added dropwise. The cells were then incubated at -20°C for 12 hours.

The cells were washed by centrifuging for 10 minutes at 200g and 4°C, removing the PBS and suspending the cells in 1 mL of PBS. The cells were then normalized to the lowest concentration of cells present in the 5 samples (1.37×10^5 cell/mL) and brought up to 1 mL with PBS. The normalization of the concentration allows for consistent staining and sample sizes for the rest of the experiment. Once all samples were equalized, the solutions were transferred to previously chilled Falcon tubes.

Propidium Iodide/Triton X-100 staining solution (2 mg DNase-free RNase A and 400 μ L of 500 μ g/mL propidium iodide brought up to 10 mL with 0.1% Triton X-100 solution in Dulbecco's PBS) was made. Cells were spun down for 15 minutes at 200 g and 4°C and the supernatant was removed. The cells were resuspended in 400 μ L of Propidium Iodide/Triton X-100 staining solution and transferred to Falcon flow cytometry tubes. The tubes were wrapped in aluminum foil and incubated at room temperature for 30 minutes. The tubes were placed back on ice and filtered into new Falcon flow cytometry tubes using nylon mesh. The nylon mesh removes any cellular clumps present. The filtered cells were then taken to the Flow Cytometry Lab at The Ohio State University for flow cytometry.

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