Decitabine treatment results into 2-5% demethylation of hypermethylated PCDH17 DMR in HCT-116 cell line in colorectal cancer

Kateryna Malkina,
Department of Biochemistry and Molecular Biology,William Conrad, Ph.D,
Lake Forest College,
Lake Forest, IL, 60045

Abstract

Colorectal cancer (CRC) has the third highest amount of diagnosis among other cancer types in the world. It is also one the cancers with the worst prognosis, taking place as the second in terms of patient death from cancer annually. Although the cell signaling pathways and molecular mechanisms behind the CRC cancer types are poorly understood, studying epigenetic modifications that potentially induce oncogenesis, such as DNA methylation, has been on the rise since it could potentially aid in identifying prominent targets of epigenetic therapy as similar DNA methylation patterns can be seen across multiple patients. Such methylated regions are called DMRs, differentially methylated regions. Research on high confidence DMRs is promising to uncover how gene silencing or activating is achieved by methylation of cytosines on the CpG sites and how it impacts cells' proliferation and survival. For this series of experiment, HCT-116 cell line from colorectal cancer was used to research the effects of a widely known demethylating cancer drug decitabine. Specifically, a DMR of interest was chosen for the study, located in the promoter region of PCDH17 gene. To prove demethylation characteristics of decitabine on HCT-116 cell line, methylation-sensitive restriction enzyme HpaII digest assay was first performed on the cells treated with various doses of decitabine and 0.1% DMSO (negative control). Then, respectful gDNA was bisulfite converted, underwent a PCR reaction, and was sent for Sanger Sequencing to analyze the percent methylation of CpG sites before and after the treatment. Although PCDH17 DMR demonstrated to be highly resistant to decitabine treatment (2-5% difference in %mC), it did overall slowdown the HCT-116 cell growth. The experimental data was uploaded to the UCSC genome browser and, in comparison with the data from the ENCODE project, demonstrated newly found CpG sites. As future studies, PCDH17 gene expression could be stimulated to test if it is silenced by the overexpressed transcription factors. It would be also useful to study this DMR and different DKO phenotypes as it might be a commonly conserved methylation that is crucial for cancer cell growth and development.

Acknowledgements

We would like to acknowledge the Teacher Assistant, Laurel Robbins, for her contribution to the reagent preparation and assistance with the experimental set up. Likewise, we would like to thank Anait Nalbandyan, Anastasia Rudan, and Leslie Garcia for being the lab partners in the experiments involving bisulfite PCR, gel electrophoresis, and colony formation assay. Finally, we are very grateful for the help, teaching, and guidance of Dr. Conrad throughout the process.

Introduction

Colorectal cancer (CRC) is one of the leading cancerous tumors in the world. It is the third most common cancer diagnosed, affecting 1.9 million people. It is also the second deadliest: it accounts for 10% of cancer mortality, counting an estimated 0.9 million deaths (Xi & Xu, 2021). Although incidence and mortality of CRC increases in adults after age of fifty, the early-onset cases, mainly of rectal cancer, have been on the increase in younger generations (Xi & Xu, 2021). Moreover, in line with predictions made by GLOBOCAN 2020, more countries are anticipated to rise in socio-economic development, leading to increase in colorectal cancer incidence as more sedentary lifestyle and change in diet take place. Thus, an outstanding 3.16 million new cases are expected to occur by 2040 (Sung et al., 2021). On a cellular level, any cancer tissue differs from a healthy cell line in a variety of morphological and functional characteristics. Such include but not limited to accelerated cell cycle, invasive growth, and cell immortality. These changes are caused by genetic mutations or epigenetics that influences gene expression without altering the DNA sequence (Cheng et al., 2019). To prevent the drastic increase in the number of cases, gene expression and its regulation in colon epithelial cells should be better understood and new screening approaches must be developed.

One of the most common epigenetic modifications that has a potential to alter gene expression is DNA methylation. This modification is known for an addition of methyl group at the C-5 position of cytosines by DNA methyltransferase (DNMT) enzymes. A genomic site where methylation usually takes place is at C-G sequence, commonly abbreviated as CpG (Lao & Grady, 2011.). As carcinogenesis occurs in a mammalian organism, methylation of cytosines in CpG islands is often found in the promoter region of tumor suppressor genes. This phenomenon is found to be correlated with transcriptional silencing in some colorectal cancer types (Lao & Grady, 2011), hence avoiding cell defense mechanisms. As for the methylation of CpG islands outside of the promoter region, it has been correlated with rather transcription activation. Since cytosine methylation plays a significant role in cancer development, it is commonly studied in the context of colorectal cancer development. According to De Carvalho et al. (2012,), cancer cells depend on DNA methylation of a few key regions for survival.

In their study, such regions were hypothesized to maintain methylation when artificially reducing global DNA methylation, such as via the knockout of DNMT3B and DNMT1 (DKO). Hence, the patterns of DNA methylation may not be random. DKO1 phenotype, for instance, is known to retain only 5% of the HCT116 wild type global DNA methylation levels, counting 566 CpG sites for 490 genes across the promoter regions. The mechanisms behind global methylation were of interest for the study conducted by Rhee et al. (2002,), where they created the DKO in the HCT-116 CRC cell line. Once both genes were absent from the cell line, they revealed that a promoter region of p16INK4a gene had lower percentage of methylated alleles. In all, it was demethylated by more than 95%. This gene turned out to be a tumor suppressor gene that got re-activated as the promoter region had less methylation. It followingly resulted in inhibition of cancer cell growth. This study was referenced to design this following series of experiments as HCT-116 cell line seemed to be sensitive to DNA methylation.

Such methylated region, a DMR, was chosen for further study on HCT-116 cells. PCDH17 gene, not known to have a role specifically in colorectal cancer, was chose for studying. The DMR is located in the promoter of the gene. It has a high confidence interval and shows to be hypermethylated in the promoter region (Simmer et al., 2012). The DMR was chosen among the 2687 frequently hypermethylated regions. As a member of protocadherin family of proteins, the role of this gene surround tumor suppression and cell to cell communication. Hence, it will be studied in the context of decitabine treatment on the HCT-116 cell line, a colorectal carcinoma cell line that was initially isolated from an adult male ("HCT116 Human Colorectal Carcinoma Reporter Gene Cell Lines - Imanis Life Sciences," n.d.).

Based on the literature information of decitabine role on the cancer cells, it was predicted to observe reduced cell growth of HCT-116 cells post treatment and demethylation of the promoter region of PCDH17 DMR. It was also anticipated to have the same effect on clonogenic survival of DKO cells as on WT based on literature from Rhee et al. (2002). The methylation sited of the gene of interest were first studied via UCSC genome browser. Then, genomic DNA (gDNA) was extracted from WT and DKO cells of HCT-116 cell line and treated with DMSO 0.1% as a negative control and various doses of decitabine as experimental conditions to identify the effect of the treatment on cell growth. Followingly, HpaII digest was performed as global demethylation assay, the gDNA was extracted, bisulfite converted, and sent for Sanger Sequencing to analyze CpG sites methylation state in PCDH17 DMR before and after treatment.

As a result of the study, decitabine demonstrated itself as effective general demethylating agent and useful for cell growth inhibition. However, it showed very little (2-5%) demethylation of the PCDH17 DMR. The Sanger Sequencing analysis revealed newly described hypermethylated CpG sites not previously included on the genome browser. Lastly, decitabine treated DKO cells showed the same pattern of clonogenic survival as WT following decitabine treatment, contrary to the fact that more than 95% of its original hypermethylation had been removed as DKO phenotype was artificially created.

Results

Decitabine treatment slows down HCT-116 cell growth

First, to determine whether a known anticancer drug decitabine decreases survival of the cells from HCT-116 cell line in colon cancer, DNA was isolated and extracted from genomic DNA (gDNA) of this cell line following the Lab 2 protocol (Conrad, 2022) and Zymo research Quick-DNA miniprep kit manufacturer instructions (Zymo Research). The detailed procedure is listed in the Methods section of this report. The respectful cells, Wild Type (WT) or Double-Knock out (DKO), were treated with decitabine (0.333ul, 0.667ul, or 1ul) or DMSO (0.1%) for two consecutive days. The cells were then counted using the hemocytometer, and the average count of cells per flask and their total volume per sample was used to estimate the total number of cells in that sample. The data displayed in Figure 1, was concluded for the WT cells treated with decitabine to have a significantly lower cell count compared to the cells treated with DMSO. Hence, it was concluded that decitabine treatment decreased cell growth for WT cells from the HCT-116 cell line. However, the DKO cells did not exhibit the same expected pattern (Figure 2). Since the analysis was split among all the lab members and class data was used to plot it, it was discussed with the instructor and the rest of the class that some groups might have had calculation error, hence result for the DKO cells cannot be interpreted.

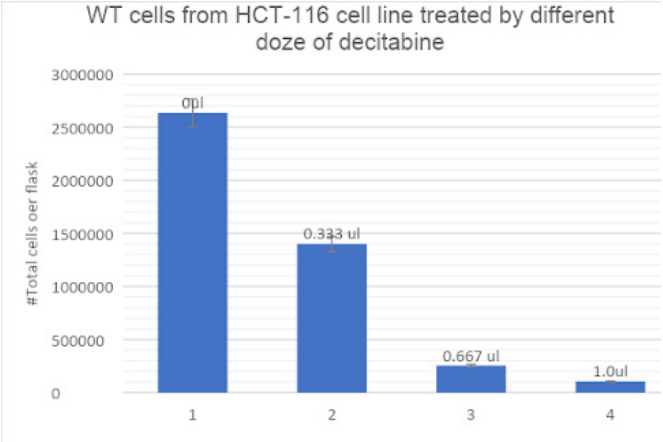

Figure 1. Decitabine at different dose (0.333ul, 0.667ul, 1.0ul) decreased WT cell growth from HCT-116 cell line. As the concentration of decitabine increased from 0 to 1.0ul, the total number of surviving cells decreased from 2633333 total cells per flask to 105000 cells. The average cells counted in a hemocytometer fluctuated from 24.5 cells to 1 cell. The cells treated with 0ul of decitabine were instead treated with DMSO (0.1%)

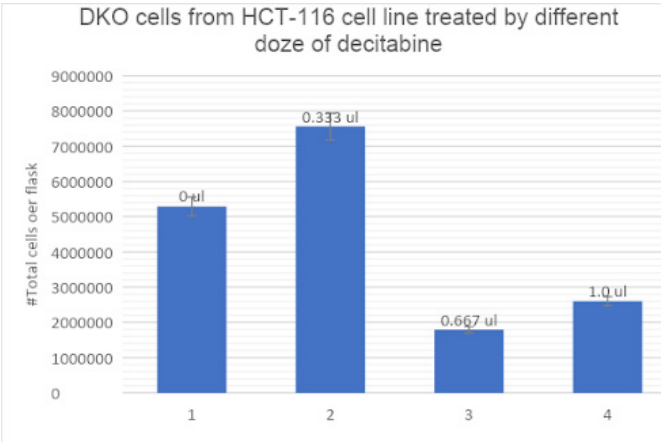

Figure 2. Inconclusive effect of decitabine at different dose (0.333ul, 0.667ul, 1.0ul) on DKO HCT-116 cell line cell growth. Due to the calculation error achieved by some of the lab members, the total number of surviving cells did not follow the expected decreasing pattern as decitabine concentration increased. The cells treated with 0ul of decitabine were instead treated with DMSO (0.1%)

HCT-116 gDNA is demethylated after decitabine treatment

The first experiment of the series demonstrated inhibition of HCT-116 cell growth after decitabine treatment. Decitabine is commonly used as an anti-cancer drug and referred to as an effective drug to decrease DNA methylation. Therefore, we tested if decitabine would demethylate DNA from previously isolated and extracted gDNA from HCT-116 cells according to the Methods section. Isolated and extracted gDNA was first ran on BioTek Synergy Spectrophotometer to assess its presence, purity, and quantity. For each sample DNA was observed in sufficient quantity and concentration in appropriate referenced range of A260/280 (>1.8), which signified DNA purity. This DNA, treated with decitabine or DMSO (- control), was then treated with HpaII or MspI restriction enzymes and gel electrophoresis was carried out with the samples to visualize demethylated bands. Among the two, HpaII is methylation sensitive, hence whether it cuts DNA or not depends on the presence of the 5-methyl group on a cytosine. Since decitabine is known to demethylate DNA, it was expected to observe more cleavage of the DNA in decitabine treated samples compared to the DMSO treated ones. Since the gel was shared among the two groups, the loading map was presented (Table 1). The loss of high molecular weight DNA in demethylated samples was observed in lanes 8 and 9 (Figure 3), which contained decitabine treated cells and HpaII enzyme. DNA degradation by the enzyme also occurs in group 2 sample 3, and slightly less in group 1 sample 3, which demonstrated that DMSO treated DNA also showed a band of demethylated DNA diminished by HpaII digest. Sample 3, DMSO treated cells, was not anticipated to show any DNA degradation for both groups since HpaII cannot cleave methylated DNA. Therefore, it could be assumed that group 2 sample 3 had contamination and group 1 sample 3 had mild contamination with demethylated DNA respectfully.

1	2	3	4	5	6	7	8	9
ladder	Sample #1 Group #1 DMSO gDNA No *HpaII*	Sample #1 Group #2 DMSO gDNA No *HpaII*	Sample #2 Group #1 Decitabine gDNA No *HpaII*	Sample #2 Group #2 Decitabine gDNA No *HpaII*	Sample #3 Group #1 DMSO gDNA *HpaII* enzyme	Sample #3 Group #2 DMSO gDNA *HpaII* enzyme	Sample #4 Group #1 Decitabine gDNA *HpaII* enzyme	Sample #4 Group #2 Decitabi gDNA *HpaII* enzyme

Table 1. Loading map of samples in an agarose gel from Group 1 and Group 2 at different conditions (DMSO/Decitabine treated), with or without HpaII enzyme treatment. Group 1 was Kateryna and Anait, Group 2 was Morgan and Anastasija. Gel electrophoresis was run at 110V for 45 minutes containing these samples in the wells.

	Concentration (ng/ul)	A260	A280	A320(raw)	A260/A28
WT DMSO (0.1%)	146.72	0.183	0.093	0.053	1.978
DKO DMSO (0.1%)	139.28	0.174	0.090	0.056	1.926
WT Decitabine (0.6)	191.84	0.240	0.122	0.049	1.961
DKO Decitabine (0.6)	189.2	0.236	0.121	0.048	1.961

Table 2. Extracted gDNA from the WT and DKO cells from HCT-116 cell line DMSO or Dectibine treated are pure (A260/280 > 1.8) and have sufficient concentration. For each condition and type of DNA, the samples were loaded and measured in BioTek UV-vis spectrophotometer to obtain concentration and purity of extracted DNA.

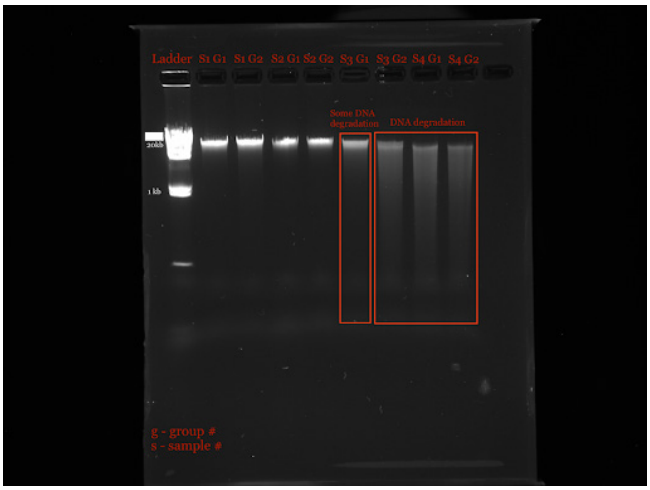

Figure 3. Decitabine demethylated (0.6ul) and DMSO (0.1%) treated genomic DNA from DKO HCT-116 cell line. The image of the gel after gel electrophoresis (110V, 45 minutes) of 8 samples from group 1 and group 2 under four unique conditions: DMSO gDNA (no HpaII), Decitabine gDNA (no HpaII), DMSO gDNA (HpaII), Decitabine gDNA (HpaII). The expected result for no enzyme present was band appearance above 20kb, seen at the lanes 2-5, estimated around 50kb. The band below 20 kb was observed for decitabine treated cells in the presence of HpaII enzyme (lanes 8, 9) and a slight smear at DMSO treated DNA in line 7 due to possible contamination as HpaII enzyme should not be able to cut methylated DNA.

DKO cells are resistant to the effect of decitabine on clonogenic survival

According to Rhee et al. (2002), in their series of experiments they did not observe a difference in clonogenic survival between WT (parental) and DKO cells following a dose curve of decitabine. This is surprising because DKO cells have >99.9% reduced methylation compared to WT, and decitabine, in part, targets DNA methylation. One possibility for why they failed to observe an effect is that decitabine has acute off-target effects and a long-term demethylating effect (Howell et al., 2010). For our set of experiments, it was decided to test if DKO cells resist the effect of decitabine on clonogenic survival when isolating the demethylating effect of decitabine. To isolate the demethylating effect of decitabine, 200 cells per well of WT and DKO cells were plated on a 6-well dish, treated with DMSO or decitabine, and then had measured the effects on clonogenic survival (see the methods section). For the expected results, if decitabine has no effect on clonogenic survival of DKO cells, then we expect to see a small decrease in the number of cells following the increase in decitabine doze with some more drastic decrease in cell survival due to a higher toxic effect of decitabine on the cell growth. However, the result is expected to match the observation for the WT cells. In turn, if decitabine decreases clonogenic survival, then we expect to see a steady decrease of the of the surviving cells with each increased doze of decitabine. It is worth mentioning that we do not expect to see decitabine increase clonogenic survival over no treatment.

As for the experimental result, we observed the first expected result, where there was some decrease in the cells post decitabine treatment as the dose of the drug increased. However, both for pretreatment and direct treatment there was a mild decrease around 100 cells maximum at as high of the decitabine doze as 150 nM. Moreover, the change in average cell count (y axis) across different dosage of decitabine (nM, x axis) for DKO cells in Figure 4 visually resembles the pattern for WT cells in Figure 5. Because we observed mild decrease in cell survival and visually similar result with the WT cells, we concluded that decitabine has no effect on clonogenic survival.

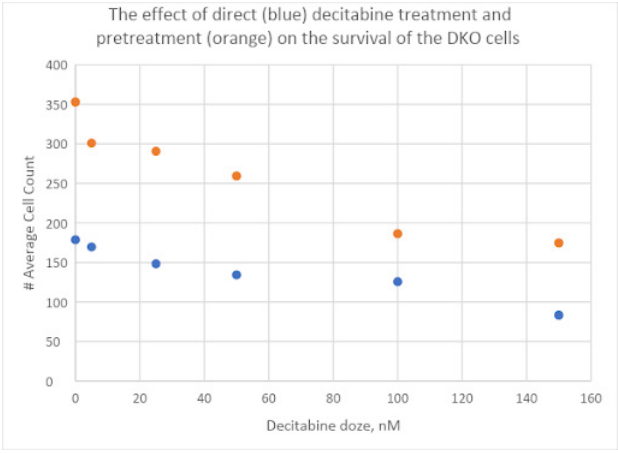

Figure 5. WT cells resist the effect of decitabine on clonogenic survival of HCT-116 colon cancer cells. Decitabine at different dose (0, 5, 25, 50, 100, and 150 nM) resulted in mild decrease in average cell count, progressively achieving lower average cell count due to overall increase in toxicity of decitabine at higher concentrations.

No amplification of gDNA extracted from HCT-116 cell line by the known primer pair FIGN

From the previous experiment it was determined that decitabine negatively impacted the cell growth from HCT-116 cell line. It is known that decitabine demethylated DNA, however, it is not known what its behavior would be like with methylated DNA in our DMR of interest. Prior to designing the primer pair specific to our DMR of interest, a positive control PCR was run on the extracted DNA with a set of primers, FIGN, know to amplify its region of interest from BIO322X course in 2020, to determine if this DNA can be amplified. The extracted DNA was first bisulfite converted and provided by the instructor to then conduct a bisulfite PCR reaction and visualize the result via gel electrophoresis. Due to time contains we were only able to run PCR reactions and prepare the gel for the gel electrophoresis. The lab instructor WHC loaded the gel and ran gel electrophoresis with our samples. We observed no DNA band in any of the lanes and some contamination on the gel around 1kb (Figure 6). Neither known DNA extracted in 2020 nor newly bisulfite converted gDNA were successful in amplification.

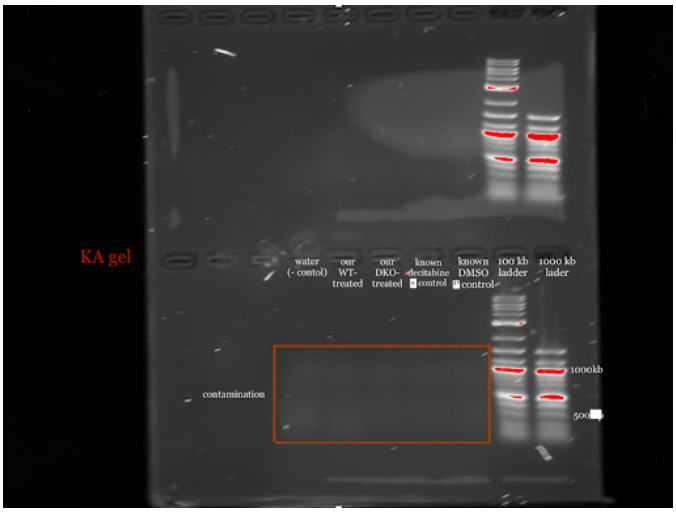

Figure 6. The newly bisulfite-converted WT and DKO-treated gDNA samples alongside known DMSO(4-) and Decitabine(4+) treated bisulfite-converted gDNA extracted in 2020 did not result in successful amplification. All the lanes, including negative control without DNA showed contamination at the amplicon length (around 500 bp) and at approximately 1kb. Two ladders were used at 100kb and 1000kb.

Promoter region of PCDH17 DMR is hypermethylated and has transcription factor activity

After concluding from the reading in class that there exist regions of the colon cancer genome, called differentially methylated regions (DMR), that are consistently hypermethylated, it was decided to identify and choose such DMR to study in HCT-116 colon cancer cells to experimentally determine if this DMR is lost after decitabine treatment. As a part of the analysis, PCDH17 gene was found nearest to a high confidence DMR. The DMR was found in the promoter region in the genome with coordinates chr13:58,202,215-58,209,939 via UCSC genome browser. The ENCODE project helped find known methylation sites in and around the promoter region and analyze the transcription factors activity in that region. Since methylation is widely known to silence gene expression, it is expected to see fewer binding of the transcription factors in the region. PCDH17 is known to be a protein coding gene that functions as a tumor suppressor gene inhibiting Wnt/beta-catenin signaling and metastasis in breast cancer and it is also frequently methylated in various cancer types, including gastric cancer (PCDH17 Protocadherin 17 [Homo Sapiens (Human)] - Gene - NCBI, n.d.). Hence, it was decided to study methylation in the promoter region of this gene in the contest of colorectal cancer cells from HCT-116 cell line. With the visualization from the UCSC genome browser, it was observed 12 transcription factors bound. Relatively to each other, the transcription factors that are most distinguishably bound were GATA2, TAF1, POLR2A, and TBP. Overexpression of GATA2 transcription factor is known to stimulate self-renewal, survival, and proliferation of cancer cells in prostate cancer (Rodriguez-Bravo et al., 2017). There is also some binding of EZH2, which is a gene function suppressor.

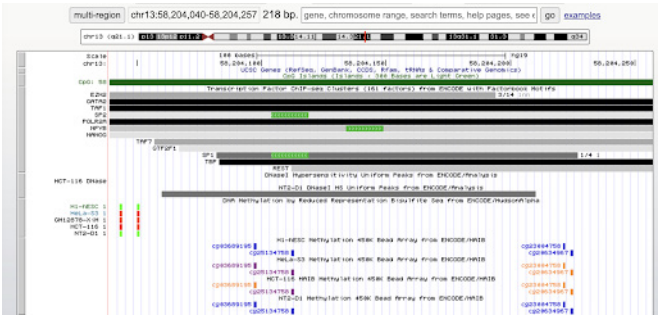

Figure 7. UCSC Genome Browser visualization of DMR in the promoter region of PCDH17 gene of interest with displayed transcription factors and known methylation sites. The UCSC genome browser screenshot of PCDH17 DMR of interest. From the top, there is a chromosome 13 position of the DMR followed by the scale of the image in nucleotide bases. This is prior to the beginning of the gene hence the start of the gene is not seen. The CpG sites in the promoter region and their quantity are indicated in green. Transcription factors and their relative to each other presence is shown in white, grey, and black colors. The darker the color, the stronger the presence of the transcription factor. From the ENCODE project there are multiple cell lines and CpG methylation sites, where red color represents 100% methylation and green represents 0% methylation.

Figure 8. UCSC Genome Browser condensed visualization of PCDH17 gene of interest with some view of the promoter region, present transcription factors and known methylation sites from the ENCODE project. The screenshot of the condensed view of the DMR of interest to display the location and orientation of PCDH17 gene on the genome.The biggest amount of transcription factors bound next to each other, as well as known methylation sites, are located at the promoter region of PCDH17 gene around 58,200,00 base pairs. Chromosome 13 position of PCDH17 followed by the scale of the image in nucleotide bases. The CpG sites in the promoter region and their quantity are indicated in green. The cell lines and CpG methylation sites in red color represents 100% methylation and green represents 0% methylation.

Optimized bisulfate PCR protocol for PCDH17 DMR amplification has twice the volume of the reverse primer and the annealing temperature of 54° C

The previous experiment aimed to amplify gDNA with a known set of primers did not result in DNA amplification as no band was seen on a gel after gel electrophoresis. Hence, for this experiment, a new set of gDNA was extracted and isolated. To study the effect of decitabine on PCDH17, the DMR of interest, forward and reverse primers were designed as a primer pair to amplify specifically this DNA region. UCSC genome browser was used to determine the coordinates of the region of interest on a chromosome that would include some of the known methylation sites, and the primer pair PCDH17 was created using the MethPrimer website accordingly (Figure 9). Once the primers were received from a manufacturer, the bisulfite converted DNA region PCDH17 was amplified with this primer pair at different annealing temperatures (52° C, 54° C, 56° C, 58° C) to determine the most suitable one for this primer pair as according to the manufacturer, the forward primer is best annealed at 52° C and the reverse at 56° C. Both WT and DKO DNA were used in this experiment, hence the most suitable temperature would ideally equally, or very similarly, amplify both DNA. The initial result showed a very faint band, that is amplification only in one well containing PCR product at 56° C annealing temperature of Wild Type DNA. In the rest of the wells, primers did not successfully align and bind to the DNA hence no amplification was observed (Figure 10).

	Primer	Start	Size	Tm	GC%	'C's	Sequence
1	Left primer	161	25	57.71	56.00	7	ATTGGTTATTTAGAAGGTTTTGGAT
	Right primer	337	24	55.60	58.33	8	AACAATTCCACCAAATAAAATAAC
	Product size: 177, Tm: 72.3, CpGs in product: 14						

Figure 9. PCD17 forward and reverse primer design on MethPrimer website. The amplicon size is labeled as product size of this primer pare at 177 nucleotides. The sequence and the start position of the forward primer is described under the “Left primer” and of the reverse primer at the “Right primer”. There are 14 CpG sites in the amplicon. MethPrimer website was used to create the primer pair and obtain the detailed information displayed on the figure.

Figure 10. Successful PCDH17 region amplification of one PCR product of Wild Type gDNA with the designed forward and reverse primer pair at 56° C annealing temperature. A band at the expected amplicon size (500 bp) observed in the 6th lane containing Wild Type DNA with annealing temperature 56° C in PCR. No amplification is observed for the samples WT (52° C), DKO (52° C), WT (54° C), DKO (54° C), DKO (56° C), WT (58° C), DKO (58° C)
.

It was decided to adjust the reagents volume in the master mix to encourage primer binding to DNA (Table 3). It was suggested by the instructor to double the volume of the reverse primer keeping the final volume constant – instead less water was added to the mix. On the second trial, all the samples except for DKO DNA at 58° C resulted in successful amplification. Successfully amplified DNA bands were observed in wells containing a PCR product of: WT (52° C), DKO (52° C), WT (54° C), DKO (54° C), WT (56° C), DKO (56° C), WT (58° C). WT at 56° C demonstrated the brightest band. The most evenly amplified gDNA from the Wild Type and DKO was at 54° C. There was no contamination in the negative control well containing no DNA.

Order of addition	Reagent	1x (ul)	5.5x (ul)
2	5x epiTaq buffer	4	22
1	diH_2O	13.0	71.5
3	10 mM dNTPs	0.4	2.2
6	Genomic DNA (8 ng/ul)	1	5.5
4	Primer F	0.5	2.75
5	Primer R	1	5.5
7	epiTaq 5 U/ ul	0.111	0.61

Table 3. Adjusted reagent table used to prepare two separate master mix solutions. For the Master Mix 1 DNA source: WT genomic DNA, Master Mix 2 DNA source: DKO genomic DNA. The temperatures tested were kept the same at 52, 54, 56, and 58° C as well as gel electrophoresis set up. The rest of the reagents' volume remained unchanged.

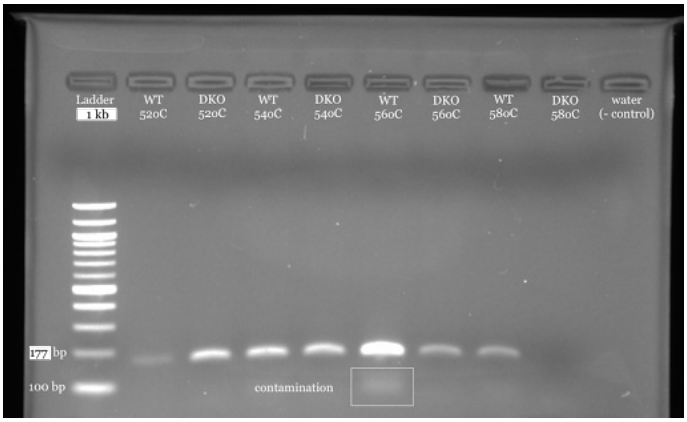

Figure 11. Successful amplification of PCDH17 region of gDNA using a designed primer pair in WT (52° C), DKO (52° C), WT (54° C), DKO (54° C), WT (56° C), DKO (56° C), WT (58° C). A band is seen at the expected size of approximate 177 bp in 7 out of 8 wells containing a DNA sample. The brightest band containing DNA product was observed in the well containing a PCR product of WT at 56° C annealing temperature. Contamination is present at around 100 bp in the same well. The two most evenly amplified bands at the same temperature but for different DNA were for an annealing temperature 54° C.

Bisulfite-converted gDNA from HCT-116 cell line amplification using designed primer pair for PCDH17 gene

Since the previous experiment demonstrated bisulfite converted gDNA amplification at 54° C annealing temperature, it was decided to use this temperature and optimized PCR reagents volume to attempt and amplify bisulfite converted gDNA from HCT116 cell line from decitabine and DMSO treated cells using designed PCDH17 primer pair. Amplification of PCDH17 region for each treatment via PCR would allow us to obtain the necessary samples to send for DNA sequencing. The sequencing would allow to further study methylation of the untreated HCT-116 cells and the effect of decitabine on demethylation state of cytosines in the CpG sites of PCDH17 DMR. The WT and DKO DNA was provided by the instructor. The PCR conducted was aimed to be at 54° C annealing temperature and among the reagents consent, the reverse primer would be added twice as much as the forward primer (5.5ul). However, while preparing the master mix, an experimental error occurred and instead 3.25ul of the reverse primer was added to the master mix 1. It was decided to keep it and rather make another master mix 2 with respectful volume of 5.5ul of the reverse primer. Gel imaging allowed to see the DNA amplicon bands occurrence at every lane, including a faint amplicon in the well containing the negative control at the expected 200bp as the amplicon size is 177bp (Figure 12). A possible source of error could be that the PCR product samples and the gel were stored over the weekend in a fridge in the laboratory, hence DNA degraded over that time. The samples could have been improperly loaded prior to gel electrophoresis hence the contamination in the no DNA well. It is challenging to make conclusions of this gel because degradation could have impacted the quality of the results. For the ladder it was also challenging to estimate the amplicon size as it was seemingly degraded. However, it is vivid that gDNA was amplified the brightest in all the wells containing wild type DNA, which could suggest that the primers anneal to and amplify wild type DNA the best. From the previous experiment, the brightest band observed on the gel image was also for a well containing wild type DNA. These samples were sent for Sanger Sequencing to the University of Chicago to further analyze CpG sites and %mC in decitabine treated and untreated cells.

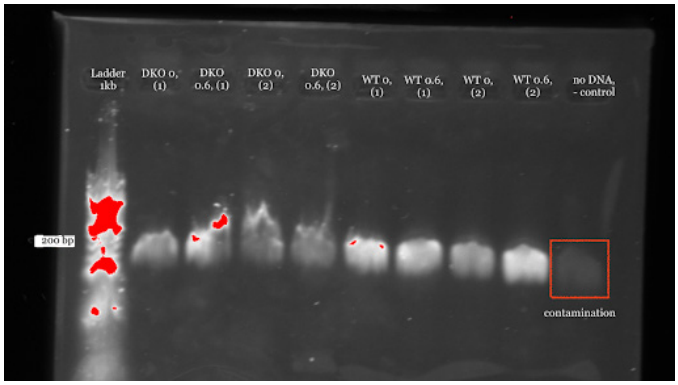

Figure 12. Amplificon of WT and DKO gDNA from HCT116 cell lines, DMSO 0.1% treated (0ul of decitabine) or decitabine treated (0.6ul) at the anticipated 200 bp amplicon size with contamination of the negative control. Lanes 2-9 containing DNA were successfully amplified with the expected amplicon size at 200 bp. The 1kb ladder, along with the amplified samples, showed DNA degradation. Contamination of the negative control in lane 10 was observed at the amplicon size 200 bp, which signifies contamination with gDNA.

Weak demethylation of PCDH17 DMR after decitabine treatment

The DMR of interest in the promoter region of PCDH17 gene was successfully amplified (Figure 12) and the samples were sent for Sanger Sequencing to the University of Chicago. The remaining methylation of the cytosines in the DKO and WT cells from the HCT-116 cell line following decitabine treatment was analyzed using the received sequence. Bisulfite conversion methodology predisposes all unmethylated cytosines to change to uracils (U), showing on the sequence data as unrecognisable (N) or thymines (T). The peak height and of the cytosines (C) in relation to the Cs and Ts was looked at as an indicator of the percent methylation (%mC.) The sequence allowed to conclude that decitabine successfully demethylated very few regions of the amplified products, not more than 5% of the initially methylated Cs. Although the same DNA was used for decitabine and DMSO treatments, two separate samples were sent for sequencing due to previously described experimental error. Prior to bisulfite PCR, the Master Mix 1 contained 3.5ul of the reverse primer (PCDH17 R), whereas the Master Mix 2 contained 5.5ul of such primer. On Figure 13, the %mC of WT DMSO treated is at 67.3% , decreasing in %mC of WT decitabine treatment to 64.2%. The same DNA that underwent the same treatment showed percent methylation of 93.2% go down to 91.2%. The reason behind different percent methylation is because having more reverse primer added most likely resulted in higher sequence quality, which allowed us to compare almost the entire bisulfite converted amplicon base sequence to the original non-treated DNA sequence.

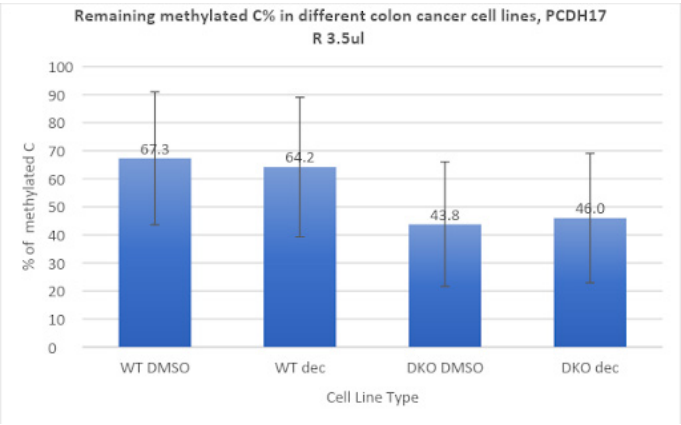

Figure 13. Decitabine treated WT cells from HCT-116 cell line resulted in 3.1% lower %mC compared to the DMSO treatment, whereas DKO decitabine treated cells showed 2.2% higher %mC that the DMSO treatment in the samples with 3.5ul of the reverse primer added. On the top of the bar graphs, calculated %mC is displayed after calculating such from the C's and T's signal strength for C's at the CpG sites using ThermoFisher VA analysis for DMSO and decitabine WT and DKO treated cells. DKO decitabine show increase in methylation % possibly due to sequence of poorer quality hence being unable to recover most of the bisulfite converted amplicon sequence. DKO is of smaller relative %mC then WT since DKO mutation already accounts for the 99.9% reduction in methylation.

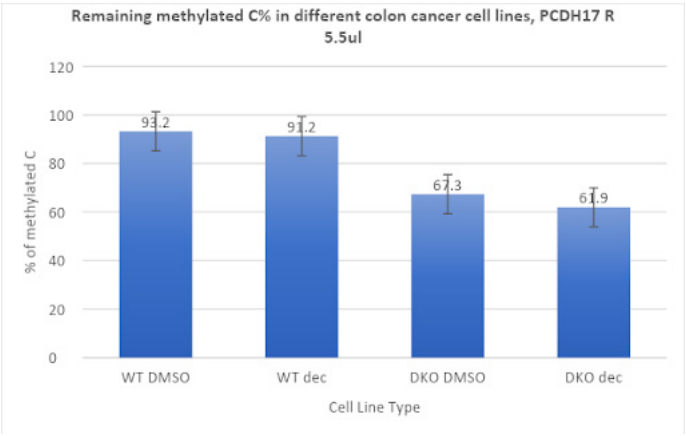

Figure 14. Decitabine treated WT cells from HCT-116 cell line resulted in 2% lower %mC compared to the DMSO treatment. Same pattern observed for DKO cells, resulting in 5.4% lower %mC following decitabine treatment. The PCR samples had 5.5ul of reverse primer added. On the top of the bar graphs, calculated %mC is displayed after calculating such from the C's and T's signal strength for C's at the CpG sites using ThermoFisher VA analysis for DMSO and decitabine WT and DKO treated cells. The WT %mC is initially higher compared to the data in Figure 13 due to higher quality sequencing data. DKO is of smaller relative %mC then WT since DKO mutation already accounts for the 99.9% reduction in methylation.

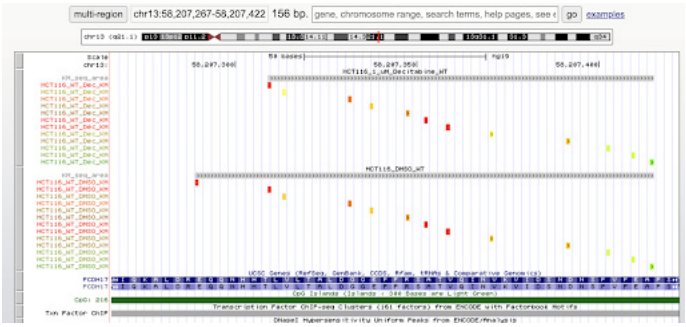

Figure 15. CpG sites not previously described in the ENCODE project found ~300 base pairs away from the known methylated regions of PCDH17 DMR for WT gDNA. The screenshot was taken of the chromosome 13 region viewed in UCSC Genome Browser with uploaded data of PCDH17 DMR that was bisulfite-converted, amplified, and sequenced. The DNA was DMSO or decitabine treated. The warmer the color is (red, orange, yellow), the higher is the methylation percent of the cytosine at that site.

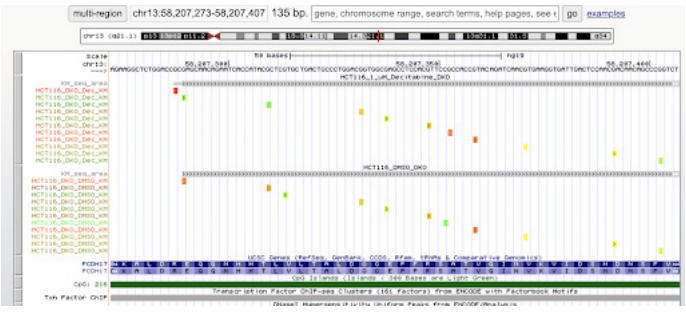

Figure 16. CpG sites not previously described in the ENCODE project found ~250 base pairs away from the known methylated regions of PCDH17 DMR for DKO gDNA. The screenshot was taken of the chromosome 13 region viewed in UCSC Genome Browser with uploaded data of PCDH17 DMR that was bisulfite-converted, amplified, and sequenced. The DNA was DMSO or decitabine treated. The warmer the color is (red, orange, yellow), the higher is the methylation percent of the cytosine at that site. More demethylated DNA (green, yellow) is observed compared to WT gDNA.

Discussion Part I

The known anti-cancer drug, decitabine, or 5-aza-2-deoxycytidine, has proven its important role in slowing down HCT-116 cell line colon cancer cell growth (Figure 1) and its ability to demethylate DNA (Figure 3). Based on the anticipated results from the early stages of the experiment, PCDH17 high confidence DMR found in the promoter region of this gene in the HCT-116 colon cancer cells was chosen for a close study. This investigation allowed us to describe newly found hypermethylated CpG sites (Figure 15, Figure 16) that could be globally hypermethylated in colon cancer in this cell line. The DMR appeared to be resistant to demethylation with and without decitabine treatment, as we observe only 2-5% decrease in methylation both in WT and DKO cells. Because PCDH17 gene plays a crucial role as a tumor suppressor (PCDH17 Protocadherin 17 [Homo Sapiens (Human)] - Gene - NCBI, n.d.), its resistance to demethylation by high doses of decitabine could be an important finding as we seek to "unsilence" the gene. Looking at the newly found methylated CpG sites (Figure 15), by zooming out it was found that their location coincides with the binding of RBBP5 and EZH2 transcription factors. This observation leads to conclusion that if not by the methylation, this gene could still be silenced by a transcription factor such as EZH2 (Figure 7) or RBP5 that regulates cell proliferation (Saijo et al., 1995). Hence, this region of interest should be studied further as it has a total of 11 transcription factors bound to it in the promoter region and more methylation sites than we previously knew from the ENCODE project. As mentioned in the Introduction, DKO1 is known to retain about 5% of the HCT116 wild type global DNA methylation levels, which corresponds to 566 CpG sites for 490 genes across the promoter regions (Carvalho et al., 2012). In our experiment, 61.9% methylation cites of decitabine treated DKO cells retained its methylated state at almost all same methylation positions (Figure 16) as found in WT (Figure 15). Hence, it can be argued that DKO1 phenotype and HCT-116 cells might share the methylation sites. Their similarity can be crucial as resistance to both decitabine demethylation and engineered double knock out that leads to global demethylation may identify this as an important target for epigenetic therapy.

Since decitabine showed to be effective only in 2-5% of this DMR demethylation, further work should be centered around understanding the mechanisms that prevent binding of the 5 present crucial transcription factors in this promoter region. According to Palii et al. (2008), the antitumor effects of 5-azadC are due to its "reactivation of aberrantly hypermethylated growth regulatory genes and cytoxicity resulting from DNA damage." Because of its resistance to decitabine and the abundance of the transcription factors at the promoter region, possible mechanisms should be investigated that intervene with reactivation of the growth regulatory genes pathways and molecules that might help regulate the cytoxicity of decitabine. Further studies can include further exploration of the methylation sites for the entire PCDH17 gene and whether there are similar methylation sites observed at the binding sites of transcription factors with similar purposes. The methylation of the DMR of interest can also be looked at on different cell lines in cancers related to the digestion system as this series of experiments was centered around the colon cancer.

Discussion Part II

The PCDH17 DMR of interest in the promoter region covers the binding area of twelve transcription factors (Figure 7). Relatively to each other, the transcription factors that are most distinguishably bound were GATA2, TAF1, POLR2A, and TBP. Besides the ones mentioned earlier, TAF1 subunit binds to core promoter sequences encompassing the transcription start site. POLR2A encodes a DNA-directed RNA polymerase II subunit enzyme that transcribes all protein-coding genes. TBP is the TATA-binding protein. There is also some binding of TAF7, which controls the first steps of transcription. When the PCDH17 gene is turned on, it produces a Protocadherin 17 protein (Figure 17) with cytoplasmic and membranous expression in most

tissues (PCDH17 Protein Expression Summary - The Human Protein Atlas, n.d.). It is potentially a calcium-dependent cell-adhesion protein involved in cell adhesion and important cell-cell connections. If methylation is observed in this promoter region, the transcription would not be possible as polymerase II wouldn't be made, TATA-binding protein would be made, and control exhibited by TAF7 on the primary steps of transcription would be disabled and no protein will be made (Figure 17) .

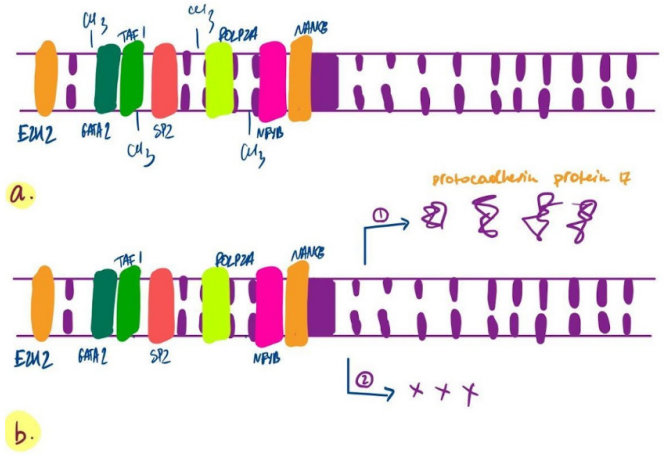

Figure 17. PCDH17 gene turned off, part a, and PCDH17 tuned on, part b. In part a, methylation of CpG sites in transcription factor binding sites leads to transcription suppression and gene silencing by inhibiting the binding of some transcription factors. In part b, transcription factor binding is possible, and protocadherin protein is synthesized, if the gene is not again silenced by the binding of EZH2 transcription factor.

Measuring gene expression is commonly achieved by quantifying levels of the gene product, and in the case of PCDH17 it would include measuring PCDH17 protein levels. One of many suitable laboratory techniques to do that would be Enzyme-Linked Immunosorbent Assay (ELISA). First, we would coat the ELISA plate with capture antibody against the antigen of interest. Anything not bound to the plate would be then washed off. Next the sample containing the cell supernatant, where protocadherin protein could be found after the cells were processed, is added to the plate. Any antigen of interest present in the supernatant would bind to the capture antibody and the rest would later be washed off the plate. Then, detection antibody labeled with an enzyme is added ready to bind to a target antigen. Finally, the substrate is added to the plate. These are commonly chromogenic and thus convert the substrate into a colored product, the intensity of which is measured by a plate reader (Enzyme-Linked Immunosorbent Assay (ELISA) | British Society for Immunology, n.d.).

Since PCDH17 promoter region has a variety of transcription factors added, including the ones that silence the gene on their own, it would be challenging to describe which signaling pathway should be activated to impact the gene expression. From literature, it is known that PCDH17 protein acts as a tumor suppressor gene inhibiting Wnt/beta-catenin signaling in some brain cancers (PCDH17 Protocadherin 17 [Homo Sapiens (Human)] - Gene - NCBI, n.d.). This signaling pathway is an evolutionary conserved mechanism that plays a role in cellular homeostasis. WNT proteins, if present in the extracellular matrix, bind to the N-terminal extra cellular cytosine-rich domain of a Frizzled family receptor, which in turn disrupts the destruction complex of beta-catenin and even triggers its cytoplasmic accumulation (Pai et al., 2017). Beta-catenin is known to promote cell-to-cell adhesion by accumulating in cell-cell contact sites. On its own, the Wnt/beta-catenin signaling is commonly overexpressed in colorectal cancers, leading to more greater tumor production (Pai et al., 2017) as beta-catenin accumulates in the cytoplasm. If the signaling pathway is de-activated in the colon cancer HCT-116 cells (Figure 18), it could result into decreased secretion of beta-catenin leading to decrease of WNT-triggered gene transcription that leads to cell proliferation. As mentioned earlier, our promoter region has overexpression of GATA2 transcription factor that is known to stimulate self-renewal, survival, and proliferation of cancer cells. If the PCDH17 gene was not initially silenced and rather involved in gene transcription,. reduced amount of beta-catenin could hypothetically prevent GATA2 transcription factor binding and thus result in reduced cell proliferation.

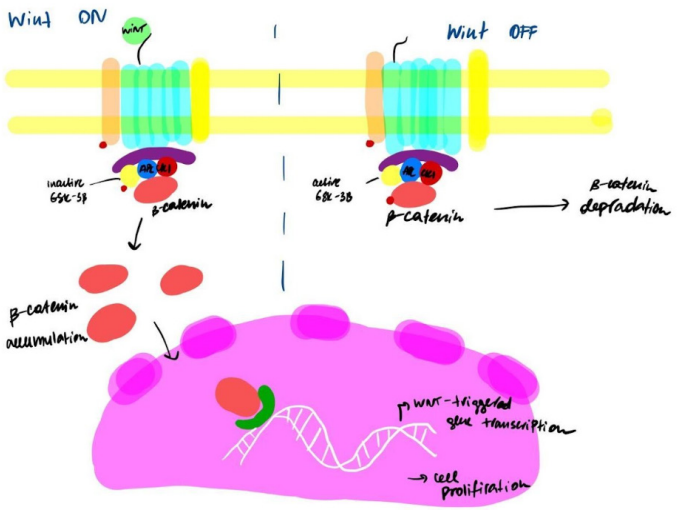

Figure 18. Change in PCDH17 gene expression following the turn off of the Wnt Signaling, resulting to no cell proliferation induced by Wnt-triggered gene transcription. If the PCDH17 wasn't initially silenced, it is likely to have the Wnt signaling turned on as it is known to be overexpressed in CRC and a transcription factor GATA2 binding site is present that is known to stimulate cell proliferation. As the signaling pathway is turned off, carcinogenic cell proliferation is anticipated to decrease.

Due to the complicated nature of PCDH17 promoter region, there are multiple potential scenarios as to how expression at the DMR would change the trait. As mentioned earlier, the methylation cites observed seem to be consistent between WT and DKO DNA. Hence, it is possible that some DKO phenotypes would still have the same silencing methylation sites. If PCDH17 gene was expressed, there are few outcomes. First, with the overexpressed presence EZH2 transcription factor in colon cancer cells, the gene can still be silenced by this transcription factor. Next, if the gene is expressed, it could lead to overexpression of the proteins that allow survival and proliferation of the cancer cells (Figure 19). Lastly, if the two transcription factors GATA2 and EZH2 are absent, the gene would result in the production of protocadherin17 protein that way participate in turning off the WNT signaling and resulting the overall destruction of excess beta catenin (Figure 18). As the result of extensive research, PCDH17 gene was found to be transcriptionally silenced across different cancer by a promoter methylation, but also inherited and somatic mutations (Berx & van Roy, 2009). In gastric cancer, for instance, somatic mutations in protocadherin family of genes can cause skipping of exon 7 or 9, which results in in-frame deletions. As a part of literature research, PCDH17 specifically has not yet been found to exabit a certain mutation in cancer that would lead to gene silencing. It is most commonly studied in the context of promoter methylation.

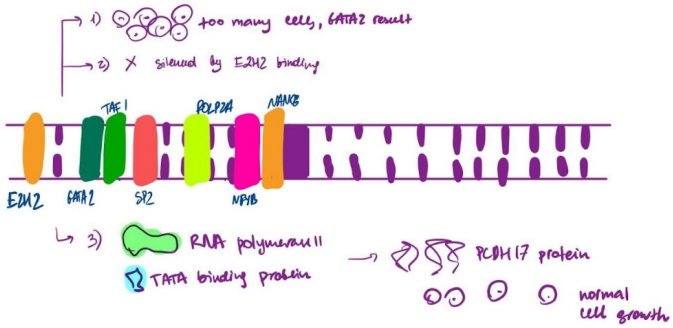

Figure 19. Three possible phenotypes as PCDH17 gene is expressed. Cancer cell proliferation (1). no effect as the gene is still silenced by the transcription factor EZH2, and normal cell growth as appropriate transcription factor binding occurs allowing to proper regulation of the transcription start by TAF1, RNA polymerase II synthesis and TATA binding protein.

PCDH17 tumor suppressor protein is secreted, and it regulates cell proliferation.

Methods

Materials

Standard molecular biology lab bench equipment, such as pipets (P20, P200, P1000) and their tips, serological pipette and pipette-aid, biohazard tip waste, liquid waste beaker, bleach, 70% ethanol, conical centrifuge tubes in sizes 15ml and 50ml, 1.5ml microcentrifuge tubes, microcentrifuge, centrifuge tube racks, kimwipes, paper towels, ultra-fine sharpies lab markers.

- Mid-log culture of HCT-116 cells
- T25 flasks
- McCoy's 5A media (complete with antibiotic)
- PBS
- Trypsin
- Hemocytometers
- Cell counters
- Trypan blue
- Compound microscopes
- 96-100% ethanol
- Not sterile Trypan Blue solution, 0.4% filtered (Sigma T8154)
- SybrSafe or ethidium bromide
- Hemocytometers
- TE buffer
- ddH2O
- BioTEK plate reader
- EZ DNA Methylation Kit
- CT Conversion Reagent
- 5 µl o'generuler 1kb DNA ladder
- Parafilm
- Mold with lid for PCR
- Narrow comb (1.0mm thickness)
- Microscopes
- Bio Rad gel doc EZ imager
- Vortex

HCT-116 Cell Line culturing and Decitabine treatment

The cells from HCT-116 cell line were cultured in 50ml McCoy's 5A media for the class by the lab instructor Dr. Conrad and the TA Laurel prior to the start of this series of experiments. At first, the pooled cells from four total mid-log cultures of HCT-116 cell line in T25 flasks were counted. Then 800,000 cells / well were transferred to 20 T25 flasks to achieve a total of 16 million cells. On the next day, either 0.1% DMSO, 0.333uM, 0.667uM decitabine, or 1uM decitabine made from 10mM stock were added to each flask. The cells were treated for 48 hours with one drug and media replacement. On the fourth day the media was replaced with no drug present. Around 72 hours later the cells were used in the first experiment. In the meantime, they were incubating.

Genomic DNA isolation, extraction, and bisulfite conversion

Genomic DNA (gDNA) isolation and extraction procedures were carried out according to W. Conrad, 2022, adapted from PureLink Genomic DNA kit (Thermo Fisher Scientific), and Zymo research Quick-DNA miniprep kit (Zymo research), using the manufacturer protocol. Deviating from the original protocol, each flask visibly containing more cells had the subsequent addition of 300ul of trypsin, whereas the ones with relatively less dense cell culture had 250uM of trypsin added to collect and count cells onwards. After tripsinizing, the cells were then counted with a hemocytometer, placing 20ul of the sample at a time in a counting chamber. The number of counted cells was used to calculate the volume of cells needed to retrieve 4 million cells total per each treatment (0uM, 0.333uM, 0.667uM, or 1uM decitabine) and cell type (WT or DKO). Genomic DNA from those 4 million cells was then purified following the procedure from the Zymo research Quick-DNATM Miniprep Kit (Zymo research). The optimized protocol had 500ul of Genomic Lysis Buffer added directly to the cell palette to meet the proportional amount 4:1 of buffer to the sample. Then extracted gDNA was eluted from the column in 100ul of the elution buffer and placed on the BioTEK plate reader to be quantified and had initial purity assessment via nanodrop UV-vis spectroscopy. Such variables as A260/280 and concentration (ng/uL) were collected per each condition. The cells from 0.333uM and 0.667uM decitabine treatment were combined among the WT and separately among the DKO for the UV-vis spectroscopy as very few cells survived the treatment.

For further investigation of the demethylation state of a DNA region of interest after decitabine treatment, the DNA had to undergo bisulfite conversion. This converted unmethylated cytosines to uraciles. EZ DNA MethylationTM Kit protocol was used to perform the conversion (Zymo Research). Based on the DNA concentration obtained with UV-vis spectroscopy, the volume of DNA needed for the DNA concentration of 400ng was calculated and separated in a PCR tube. After the following addition of 5ul M-dilution buffer and ddH2O to bring the final volume to 50ul, the solution was incubated for 15 minutes at 37oC. Then, 100ul of freshly prepared CT Conversion Reagent was added to the solution and it was place in a thermal cycler for incubation in the dark at 50oC for 12-16 hours. It was followed by the second round of incubation at 0-4oC for 10-20 minutes. Afterwards the sample was places in Zymo-SpinTM IC column with 400ul of M-Binding Buffer and centrifuges at full speed (>10,000xg) for 30 seconds. Discarding the flow-through was followed by a total of alternative additions and centrifugation with M-Wash Buffer, M-Desulphonation Buffer, M-Wash Buffer, and M-Elution buffer.

Clonogenic Survival Assay

To measure immortality of HCT-116 cells a clonogenic survival assay was performed according to Palii et al. (2008). Briefly, for direct treatment approximately 200 HCT-116 WT and DKO cells were passaged into 6-well tissue culture plates. After overnight incubation, cells were treated with the following doses of decitabine in 0.1% DMSO (final ; v/v) for 48 hrs with one drug replacement (0, 5, 25, 50, 100, and 150 nM decitabine (sigma part# A3656.) After an additional 14days of growth, cells were fixed and stained (1x phosphate buffered saline (sigma part D1408); 6% glutaraldehyde (v/v; EMS part 16220); and 0.5% crystal violet (w/v; sigma part 61135)) for one hour. Cells were immersed repeatedly in excess tap water until colonies were visible. Colonies were counted by eye (Conrad, 2022).

Global demethylation assay

To test for DNA demethylation of HCT-116 cells by decitabine treatment, a procedure with digest enzymes HpaII (NEB R0171S) and MspI (NEB R0106S) was performed according to the lab manual in Lab 3 (Conrad, 2022). A solution contained the necessary volume of 400ng gDNA calculated using previously extracted gDNA concentration data, cutsmart buffer, 0ul (the negative control) or 0.5ul of one of the two restriction enzymes, and diH2O. The solution then underwent a bisulfite PCR reaction. PCR products were then analyzed by gel electrophoresis. Out of the two restriction enzymes, HpaII enzyme is the only sensitive to methylation. Both recognize the CCGG sequence and cleave DNA, but cleavage by HpaII would be prevented by the presence of a 5-methyl group on a cytosine (Waalwijk & Flavell, 1978).

Bisulfite Polymerase Chain Reaction (PCR)

A bisulfite PCR reaction was carried out according to the EpiMark Hot Start Taq DNA Polymerase Guidelines for PCR (M0490) (New England Biolabd inc.) and optimized protocol sourced from the Lab manual (Conrad, 2022). PCR was used to examine amplification of the gDNA samples in each respectful experiment. The extracted gDNA samples, Wild Type and DKO, for both DMSO and Decitabine treatments were added to their respectful Master Mixes. The central components of a Master Mix for the PCR reactions contained: 5x epiTaq buffer, diH2O, 10 mM dNTPs, bisulfite-specific forward and reverse primers (10uM each), and epiTaq 5 U/ ul enzyme that is a DNA polymerase. The PCR reactions were set at different temperatures (Table 5) for about 1 hour running a total of 39 cycles. The reagents concentration is kept constant in the master mix to have sufficient reagents to perform 5.5x reactions. To achieve this, the initial volume of reagents needed for 1x reaction was adjusted accordingly preserving the initial concentration of the reagents. Each PCR reaction had either a different DNA sample, annealing temperature, or the primer pair used. Each of the differences indicated in the figure legends in the Results section. The primer pair to amplify PCDH17, the DMR of interest, was designed using the MethPrimer website tools.

Order of addition	Reagent	1x reactions (ul)	5.5x reactions (ul)
2	5x epiTaq buffer	4	22
1	diH_2O	13.5	74.25
3	10 mM dNTPs	0.4	2.2
6	Genomic DNA (8 ng/ul)	1	5.5
4	Primer F (10 uM)	0.5	2.75
5	Primer R (10 uM)	0.5	2.75
7	epiTaq 5 U/ ul	0.111	0.61

Table 4. Master Mix reagents table with indicated volume per each reagent to carry out 1x or 5.5x reactions.

PCR reaction type	Annealing Temperature(s)
HpaII restriction enzyme PCR	56°C
Positive control amplifying DNA with FIGN primers PCR	56 °C
Optimizing temperature for amplifying DNA with PCDH17 designed primer pair	52 °C, 54 °C, 56 °C, 58 °C
PCDH17 primer pair PCR	54°C

Table 5. Annealing temperature conditions for each PCR reaction carried out in this set of experiments.

Gel Electrophoresis

Following the PCR reactions, gel electrophoresis was performed according to the adapted procedure from the DOE JGI "Genomic DNA QC Using Standard Gel Electrophoresis (for collaborators)" (Lin, 2012). It was used to visualize and assess the quality of gDNA amplification from DMSO and decitabine treated HCT-116 cells. Agarose gel was prepared in 250 mL Erlenmeyer flask weighing out 0.5g of agarose and 50 ml of 1x TBE buffer. Microwaving on the defrost setting for 30 seconds dissolved the agarose in the buffer. The solution was then cooled down with the water with the subsequent addition of 5uL ethidium bromide or SybrSafe and swirling to mix the solution. It was poured into a mold to solidify with a narrow 1.0mm thick comb creating 10 wells and left to solidify. In the meantime, 5ul of 6x DNA loading buffer was added to the PCR samples. 1x TBE buffer was used to fill the rest of the space surrounding the gel and the samples were loaded in the gel. The gel electrophoresis run for around 45 minutes and imaged on a Bio Rad gel doc EZ imager to visualize amplified bands.

Quantifying DNA methylation

Analysis of the gDNA sequencing was performed according to the procedure listed in the SOP for Lab 11 (Conrad, 2022). The samples containing amplified gDNA with self-designed PCDH17 primer pair and experimentally determined optimum annealing temperature had 1ul ExoI and 2ul rSAP added and were submitted for Sanger sequencing at the University of Chicago. The sequence analysis was centered around methylated CpG sites and quantifying the percent methylation of the cytosines (%mC) for these sites at previously chosen PCDH17 DMR. The data containing peaks at different nucleotides was first visualized via the Thermo Fisher Cloud Variant Analysis app. Then, Emboss Needle tool was used to align the nucleotide sequence data with the sequence from the initial amplicon of choice at the known nucleotide positions (EMBL-EMBI). This alignment was used to determine the CpG sites location. Using Microsoft Excel, %mC was calculated from the signal strength of cytosines in ratio with the sum of the signal strengths of cytosines and thymines at the CpG sites. The formula used was %mC=(C/(C+T))×100% to obtain the final value in percentage. The %mC of DMSO and decitabine treated samples were compared. Analyzed data was uploaded on the UCSC Genome Browser in alignment with PCDH17 DMR to compare observed CpG sites with the known methylation regions this DMR.

References

Berx, G., & van Roy, F. (2009). Involvement of members of the cadherin superfamily in cancer. Cold *Spring Harbor perspectives in biology*, 1(6), a003129.

Conrad, W. (2022). Lab 1: Agenda and instructor notes, BMB322X Lab Archives.

Conrad, W. (2022). Lab 2: Agenda and instructor notes, BMB322X Lab Archives. Collecting and lysing cells for genomic DNA extraction.

Conrad, W. (2022). Lab 3 SOP and intro: gDNA quality control and HpaII digest. BMB322X Lab Archives.

Conrad, W. (2022). Lab 4 SOP: clonogenic survival assay. BMB322X Lab Archives.

Conrad, W. (2022). Lab 6: SOP Bisulfite treatment of genomic DNA. BMB322X Lab Archives.

Conrad, W. (2022). Lab 7 SOP: Running a PCR. BMB322X Lab Archives.

Cheng, Y., He, C., Wang, M. et al. (2019) Targeting epigenetic regulators for cancer therapy: mechanisms and advances in clinical trials. *Sig Transduct Target Ther 4, 62.*

De Carvalho, D. D., Sharma, S., You, J. S., Su, S. F., Taberlay, P. C., Kelly, T. K., Yang, X., Liang, G., & Jones, P. A. (2012). DNA methylation screening identifies driver epigenetic events of cancer cell survival. *Cancer cell*, 21(5), 655–667.

EMBOSS Needle < Pairwise Sequence Alignment < EMBL-EBL. (n.d.). *EMBL-EBL*.

Enzyme-linked immunosorbent assay (ELISA) | British Society for Immunology. (n.d.). Retrieved December 15, 2022.

EpiMark® Hot Start Taw DNA Polymerase Guidelines for PCR (M0490) | NEB. (n.d.). *New England BioLabs Inc*.

EZ DNA Methylation Kits. (n.d.). *Zymo Research.*

HCT116 Human Colorectal Carcinoma Reporter Gene Cell Lines—Imanis Life Sciences. (n.d.). *Imanis Life Sciences | United States*. Retrieved December 15, 2022, from

Howell, P. M., Liu, Z., & Khong, H. T. (2010). Demethylating Agents in the Treatment of Cancer. *Pharmaceuticals*, 3(7), 2022–2044.

Lao, V. V., & Grady, W. M. (2011). Epigenetics and colorectal cancer. Nature reviews. *Gastroenterology & hepatology*, 8(12), 686–700.

Lin, P. (2012). Genomic DNA QC using standard gel electrophoresis (For Collaborators). *Doe Joint Genome Institute,* V3.

Palii, S. S., Emburgh, B. O. V., Sankpal, U. T., Brown, K. D., & Robertson, K. D. (2008). DNA Methylation Inhibitor 5-Aza-2′-Deoxycytidine Induces Reversible Genome-Wide DNA Damage That Is Distinctly Influenced by DNA Methyltransferases 1 and 3B. *Molecular and Cellular Biology*, 28(2), 752–771.

Pai, S. G., Carneiro, B. A., Mota, J. M., Costa, R., Leite, C. A., Barroso-Sousa, R., Kaplan, J. B., Chae, Y. K., & Giles, F. J. (2017). Wnt/beta-catenin pathway: Modulating anticancer immune response. *Journal of Hematology & Oncology,* 10(1), 101.

PureLinkTM Genomic DNA Mini Kit. (n.d.). *Thermo Fisher Scientific.*

PCDH17 protein expression summary—The Human Protein Atlas. (n.d.). Retrieved December 15, 2022.

PCDH17 protocadherin 17 [Homo sapiens (human)]—Gene—NCBI. (n.d.). Retrieved December 15, 2022.

Quick-DNATM Miniprep Kit. (n.d.). *Zymo Research.*

Rhee, I., Bachman, K. E., Park, B. H., Jair, K.-W., Yen, R.-W. C., Schuebel, K. E., Cui, H., Feinberg, A. P., Lengauer, C., Kinzler, K. W., Baylin, S. B., & Vogelstein, B. (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*, 416(6880), 552–556.

Rodriguez-Bravo, V., Carceles-Cordon, M., Hoshida, Y., Cordon-Cardo, C., Galsky, M. D., & Domingo-Domenech, J. (2017). The role of GATA2 in lethal prostate cancer aggressiveness. *Nature Reviews Urology,* 14(1), 38–48.

Saijo, M., Sakai, Y., Kishino, T., Niikawa, N., Matsuura, Y., Morino, K., Tamai, K., & Taya, Y. (1995). Molecular Cloning of a Human Protein That Binds to the Retinoblastoma Protein and Chromosomal Mapping. *Genomics*, 27(3), 511–519.

Simmer, F., Brinkman, A. B., Assenov, Y., Matarese, F., Kaan, A., Sabatino, L., Villanueva, A., Huertas, D., Esteller, M., Lengauer, T., Bock, C., Colantuoni, V., Altucci, L., & Stunnenberg, H. G. (2012). Comparative genome-wide DNA methylation analysis of colorectal tumor and matched normal tissues. *Epigenetics*, 7(12), 1355–1367.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: *A Cancer Journal for Clinicians*, 71(3), 209–249.
Waalwijk, C., & Flavell, R. A. (1978). MspI, an isoschizomer of hpaII which cleaves both unmethylated and methylated hpaII sites. *Nucleic acids research*, 5(9), 3231–3236.
Xi, Y., & Xu, P. (2021). Global colorectal cancer burden in 2020 and projections to 2040. *Translational Oncology*, 14(10), 101174.