Decitabine treatment demethylates vast majority of high-confidence differentially methylated regions in HCT-116 colorectal cancer cells [version 1; peer review: 2 not approved]

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Abstract

Background: Gene silencing by CpG island hypermethylation often plays a role in colorectal cancer (CRC) progression. Certain regions of the genome, called high confidence differentially-methylated regions (DMRs), are consistently hypermethylated across numerous patient samples.

Methods: In this study, we used bioinformatics and bisulfite PCR sequencing of HCT-116 cells to investigate methylation levels at DMRs in the promoters of six genes: DKK3, EN1, MIR34b, SDC2, SPG20, and TLX1. We then investigated whether the anti-cancer drug decitabine, had a demethylating effect at these promoter regions.

Results: We found that hypermethylation correlated with lack of transcriptional enhancer binding in these six regions. Importantly, we observed that for all DMRs, decitabine significantly reduced CpG methylation. Decitabine also reduced clonogenic survival, suggesting that there is a correlation between lower CpG island methylation levels and reduced cancerous properties.

Conclusions: Our study provided single-nucleotide resolution and revealed hypermethylated CpG sites not shown by previous genome-wide methylation studies. In the future, we plan to perform experiments that demonstrate a causal link between promoter hypermethylation and carcinogenesis and that more accurately model treatments in CRC patients.

Keywords
epigenetics, CpG methylation, colon cancer, decitabine
Introduction
Colorectal cancer (CRC) is the third most common type of cancer worldwide, yet it is often caught only in its late stages\(^1\). While CRC incidence has been decreasing for individuals above 50 years of age, the incidence rates increased by 22% between 2000–2013 in individuals below 50\(^2\). Chemotherapy is not effective, and patients typically survive only 44 months after the completion of treatment\(^3\). Despite this pressing reality, there is still debate about the molecular mechanisms underlying this disease. Genetic and epigenetic factors contribute to oncogenesis, but there is no consensus on a definitive molecular pathway\(^4\).

Cytosine methylation changes the expression of genes involved in cancer progression. DNA methylation is the transfer of a methyl group onto the C5 position of cytosine to form 5-methylcytosine\(^5\). Promoter hypermethylation has been observed to drive CRC disease progression. For instance, promoter hypermethylation of the hMLH1 gene leads to mismatch repair defects and a hypermutator phenotype in CRC\(^6\). Genetic knockout of two DNA methyltransferase genes restores expression of the tumor suppressor gene CDKN2A and slows growth of the CRC cell line HCT116\(^7\).

Numerous genomic regions have been shown to be consistently hypermethylated in multiple samples of colon cancer in comparison to regular colon cells\(^8\). For instance, Simmer et al.\(^9\) found 2867 genomic regions consistently differentially methylated regions in CRC (DMRs). However, the genome-wide methylation techniques used by these studies lack nucleotide-level resolution at these DMRs\(^10\), and do not address if these DMRs play a mechanistic role in CRC progression.

We sought to investigate if the colorectal cancer cell line, HCT-116, can serve as a viable model for DMR hypermethylation. HCT-116 cells have been observed to be an orthotopic model for colon cancer in mice\(^11\). Furthermore, knockout of the DNA methyltransferases DNMT1 and DNMT3b has been observed to deplete >98% of total methylation and slow HCT-116 growth\(^12\), indicating global DNA methylation plays a role in HCT-116 cell survival. If HCT-116 cells are to serve as a model for DMR hypermethylation in CRC, then we expect to observe hypermethylation at established high-confidence DMRs and a reversibility of methylation with the demethylating anticancer drug, decitabine\(^13\).

In the present study, we sought to investigate the effect of the decitabine on high confidence DMRs found in the promoter regions of genes previously connected to cancer progression from 2648 available DMRs. We selected DMRs proximal to DKK3, EN1, mir34b, SDC2, SPG20, and TLX1. DKK3 is a Wnt signaling pathway inhibitor found to be hypermethylated in colon cancer cells, possibly promoting oncogenic Wnt signaling\(^14\). EN1 is a canonical gene in development\(^15\) but has recently been linked to increased cell proliferation as a non-canonical prosurvival transcription factor in cancer cells\(^16\). In addition, the EN1 promoter is found to be hypermethylated in CRC with a CpG island methylator phenotype\(^5\). mir34b is a micro-RNA that is essential for normal brain development, motile ciliogenesis and spermatogenesis\(^17\). It was found to be hypermethylated in 100 out of 101 colon cancer cell lines\(^18\). SDC2 is a transmembrane proteoglycan that mediates cytoskeletal organization and adhesion to the ECM\(^19\). SDC2 acts as a positive regulator of growth factor signals whose aberrant expression correlates with tumor size\(^20\). SDC2 promoter is hypermethylated in cancer\(^21\). SPG20 regulates cytokinesis and may alter cell division when aberrantly methylated in CRC\(^22\). TLX1 functions as a transcription factor\(^23\) and its gene promoter is frequently hypermethylated in CRC\(^24,25\). While it is overexpressed and demethylated in leukemia\(^26\), aberrant hypermethylation may also promote growth, as reviewed in 25.

We predicted that decitabine will have a negative effect on HCT-116 proliferation, and will decrease hypermethylation at these DMRs. Using the UCSC genome browser, we observed that DMR methylation inversely correlated with transcriptional activator binding across multiple cell lines. Using bisulfite PCR we observed that decitabine inhibits methylation across each DMR.

Methods
Transcriptional enhancer binding analysis
DMRs were selected from Simmer et al.\(^26\). Genomic regions from table S4 of that publication were aligned to human genome 19 (hg19) using liftOver\(^27\). UCSC genome browser was configured to visualize transcription factors and methylation status according to the following configuration: http://genome.ucsc.edu/s/williamhconrad/hg19%20DaIl%20Deel%20Dmethylation. Transcription factors were selected randomly from the track UCSC genome browser track “Transcription Factor ChiP-seq (161 factors) from ENCODE with Factorbook Motifs”. 10 cell lines with transcription factor binding were recorded and 10 cell lines lacking transcription factor binding were recorded at each DMR. Cell lines were selected using a random number generator. Each transcription factor was recorded as a transcriptional repressor or enhancer according to UniProt\(^27\). The methylation status was then recorded for each of those cell lines. Methylation was recorded as fully methylated, mostly methylated, majority methylated, half-methylated, minority methylated, mostly unmethylated, fully unmethylated, not detected, or not tested. The cell lines were then sorted into methylated or unmethylated. The number of repressors and enhancers was recorded, and a chi squared analysis was performed to compare differences in repressor and enhancer binding to methylated or unmethylated regions using excel Data are published as underlying data\(^28\).

HCT-116 cell culture
HCT-116 cells were obtained from American type culture collection (ATCC; CCL-247) and cultured in McCoy’s 5A media (ATCC; 30-2007) in 10% fetal bovine serum (ATCC; 30-2007) and penicillin streptomycin (Thermo; 15140122). Cells were maintained in tissue culture flasks at 37 °C and 5% CO\(_2\) according to established protocols from ATCC\(^29\).

Clonogenic survival assay
To test the effect of decitabine on the clonogenic survival of HCT-116 cells, we used protocols adapted from Franken...
et al. and Palii et al. Cells were plated at a density of 200 per well in a 6-well tissue culture plate. After overnight incubation, cells were treated with vehicle (anhydrous DMSO; Sigma; 276855), 0.1, 0.25, or 1 µM decitabine (Sigma; A3656). Cells were treated again after 24 hrs and then replaced with 2 ml of complete McCoy’s 5A media. After 12 days of growth, cells were fixed and stained with 10% w/v glutaraldehyde (Sigma; 340855), 0.5% w/v crystal violet (Sigma; C6158) in PBS (Sigma; 1408). After extensive rinsing in tap water, colonies were counted by eye. The average number of colonies was compared across the four conditions using a one-way ANOVA followed by Tukey’s HSD post hoc test.

Decitabine treatment and genomic DNA isolation
HCT-116 cells were cultured as described above. Exponentially growing cells were passaged to a density 20% confluency. The day following passage, cells were treated with 1 µM decitabine or DMSO. After 24 hours, treatment was repeated. After four additional days of incubation, cells were collected by trypsinization. 400,000 cells for each condition were collected and genomic DNA was isolated using the PureLink genomic DNA mini kit according to the manufacturers instructions (Thermo Fisher; K182001).

Global demethylation analysis
400 ng of genomic DNA isolated as above was bisulfite converted using the EZ DNA methylation kit according to the manufacturer’s instructions (zymo research; D5014). Primer pairs (Table 1), optimal annealing temperatures (Table 1), and PCR reaction conditions (Table 2), reaction master mix (Table 3) are provided in the indicated tables. Amplified DNA was purified from oligonucleotide primers and dNTPs using zymo DNA clean and concentrator-5 according to the manufacturer’s instructions (zymo; D4013). Samples were eluted in 10 µl of elution buffer and submitted for sequencing at the University of Chicago Comprehensive Cancer Center DNA sequencing and genotyping facility (Chicago, IL). Percent methylation was calculated using the relative peak height of cytosine and uracil at a given CpG site, as described previously. Peak height was quantified using Thermo Fisher Variant analysis app on the thermo fisher connect web site. Briefly, this cloud-based application processes .abi sequencing chromatogram files and returns base calls and peak height values for each peak on the chromatogram. The open source software Chromaseq can also extract identical base call and peak height values from .abi files. Briefly,

| Table 1. Primers used in this publication. |
|-------------------------------------------|
| **DMR** | **+ or - strand** | **Primer sequence** | **Forward or reverse sequencing primer?** | **Annealing temp (C)** |
| DKK3 | negative | TAAAGAAGGTGTAGTATAGGT | F | no | 56 |
| DKK3 | negative | ACTATTAAACCTAAAAACCAAAA | R | yes | 56 |
| TLX1 | positive | TTTTTAGTTTTGTAGTTTTTTT | F | no | 52 |
| TLX1 | positive | AAACCTCAAATACCTAAACCCC | R | yes | 52 |
| mir34b | positive | TTATAGTATAATGTTAATGATAGTTTT | F | no | 52 |
| mir34b | positive | AACACTCCTAAAAACCTAAAAATC | R | yes | 52 |
| EN1 | positive | TAGGTTAAGTTAGGGTTAGGAA | F | no | 44 |
| EN1 | positive | AAAACTCCCTATTCAACACTTAA | R | yes | 44 |
| SPG20 | positive | TATTAAAAAGTTAAGGTTAGGAGGT | F | no | 52 |
| SPG20 | positive | CCCAATATAAATATAAAAAACTAATCC | R | yes | 52 |
| SDC2 | positive | ACCAAACCCAAATACACCAAATC | F | no | 52 |
| SDC2 | positive | TTTTTATTTTTGTATGGTTATT | R | yes | 52 |
| CDKN2A | positive | GTGAGGGTTAGGAGGAGTAG | F | no | 52 |
| CDKN2A | positive | TAACTATACCTATTCCAAAATACC | R | yes | 52 |
chromaseq can be installed according to their web site. The .abi sequencing files can be viewed using this software. Selecting a base call will reveal the identical peak height value presented in the chromatogram as exported in the Thermo Fisher Variant analysis app.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism version 7.0d. To evaluate the effect of various doses of decitabine on clonogenic survival against a no-decitabine control, a one-way ANOVA with Tukey’s post hoc was performed to control for multiple comparisons (i.e. all drug conditions against the same control). To evaluate the percent methylation of DMRs in the presence or absence of decitabine, a one-way ANOVA was performed with Bonferroni’s post hoc test to allow for multiple comparisons (i.e. between control and decitabine treated for each DMR). The specific statistical tests used are also described in the figure legends.

**Results**

**Methylation inversely correlates with transcriptional enhancer binding**

In mammals, cytosine methylation inversely correlates with gene expression. We sought to determine if our selected DMRs might regulate gene expression. Using the UCSC genome browser, we identified transcription factor binding data in our selected DMR regions from the encyclopedia of DNA elements (ENCODE) project. The ENCODE project has performed 2041 transcription factor CHIP-seq experiments across 90 cell lines. Transcription factor binding was observed at our DMRs (Figure 1). We hypothesized that if methylation silenced gene expression at the DMRs we selected, then we would see diminished binding of transcriptional enhancers in cell lines with methylation present at that DMR. For each of our six DMRs we observed TF binding across 20 cell lines selected by random number generator (see methods). We categorized the degree of TF binding and degree of methylation for these 10 cell lines (Underlying data for Table 4).

Indeed, we observed no transcriptional enhancers bound to methylated DMRs. Interestingly, binding of transcriptional repressors was also diminished at methylated DMRs, perhaps because methylation supplants the need for transcription factor repression. In general, transcriptional repressors and enhancers both bound more readily to cell lines with unmethylated DMRs (Table 4). The differences between repressor and enhancer binding in methylated and unmethylated DMRs was significantly different by chi-squared analysis (p<0.05). From these data, we conclude that methylation of the selected DMRs repress transcriptional enhancer binding across a broad range of cell lines.

**Decitabine diminishes clonogenicity of HCT-116 cells and decreases global DNA methylation**

The ability of a cancer cell to form a colony has been a long-standing measure for its survival in the host. Interventions that ablate clonogenicity increase patient survival. Decitabine is known to inhibit clonogenic survival of HCT-116 cells, and we observe inhibition of clonogenic survival at similar doses ranging from 1 µM to 100 nM (Figure 2). Furthermore, decitabine treatment is known to increase sensitivity of HCT-116 genomic DNA to the restriction enzyme, HpaII, which is inhibited by CpG methylation. Likewise, we observe that genomic DNA collected from HCT-116 cells treated for 48 hr with 1 µM decitabine was digested by decitabine (Figure 3).

From these data, we conclude that decitabine inhibits the clonogenic survival of HCT-116 cells and also inhibits DNA methylation.

**Methylation at selected DMRs is decreased by decitabine**

After observing global demethylation by 1 µM decitabine (Figure 3), we next sought to determine the degree of CpG methylation at DMRs in HCT-116 cells, and if those methylation sites were inhibited by decitabine. Using bisulfite PCR, we detected conversion of unmethylated cytosine to uracil (Figure 4a). Importantly, we both identified methylated CpG sites previously detected by reduced-representation bisulfite sequencing as part of the ENCODE project, and we also identified novel CpG sites in the region, adding resolution to the methylation status of these select DMRs (Figure 4b). We quantified the degree of CpG methylation at each site detected by bisulfite PCR in the presence or absence of decitabine. We observed statistically significant reductions in CpG methylation at all tested DMRs. From these data we can conclude that all colon cancer DMRs tested are hyper-methylated in HCT-116 cells, that bisulfite PCR offers

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**Table 2. PCR reaction protocol for Labnet TC9610 Multigene Optimax thermal cycler.**

| step | cycle | temp (°C) | time (m:ss) |
|------|-------|-----------|-------------|
| 1    | denaturing | 95 | 5:00 |
| 2    | denaturing | 95 | 0:30 |
| 3    | annealing | See Table 1: | 0:45 |
| 4    | extension | 68 | 0:45 |
| 5    | go to step 2 39 times |

**Table 3. PCR reagents.**

| Reagent                  | vol (ul) |
|--------------------------|----------|
| 5x epimark Taq buffer    | 4        |
| (New England Biolabs; M0490S) |
| dH2O                     | 13.5     |
| 10 mM dNTPs              | 0.4      |
| (New England Biolabs; N0447S) |
| genomic DNA (8 ng / ul)  | 1        |
| Primer F (10 uM)         | 0.5      |
| Primer R (10 uM)         | 0.5      |
| Epimark Taq 5 U/ ul      | 0.111    |
| (New England Biolabs; M0490S) |
**Figure 1.** UCSC genome browser view of Transcription factors bound to the DKK3 DMR. Representative screen capture of the UCSC genome browser view of the DKK3 DMR. Scale depicted in track 1 (black). DKK3 gene depicted in track 2 (blue). Transcription factor binding observed in ENCODE database depicted in track 3 (grey). CpG islands depicted in track 4 (green). CpG methylation as observed by reduced representation bisulfite sequencing in HCT-116 cells depicted in track 5 (red is 100% methylated, green is 0% methylated).

**Table 4. Methylation at selected DMRs inversely corresponds to enhancer binding.** At each DMR, transcription factor binding was evaluated across cell lines tested in the ENCODE project. No transcriptional activators were detected at methylated DMRs across cell lines. Fewer repressors were also detected at methylated DMRs. However, repressors failed to bind methylated and unmethylated DMRs with similar frequency.

| Repressor binds | Enhancer binds |
|----------------|----------------|
| methylated     | Y   N   Y   N |
| unmethylated   | 21  8  33  22 |

**Figure 2.** 1 μM Decitabine inhibits clonogenic survival. Number of HCT-116 colonies detected by crystal violet staining 14 days after two 24-hour treatments of the indicated doses of decitabine. *p<0.05 ANOVA, Tukey’s post-hoc.

**Figure 3.** Global DNA methylation is reduced by decitabine. HCT-116 cells were treated with 1 μM decitabine or DMSO for 48 hrs. Genomic DNA was extracted and treated with the methylation-sensitive enzyme, HpaII as indicated. 400 ng of gDNA was separated by gel electrophoresis. Ladder indicated to the left of the gel.

Discussion

From our results, we conclude that HCT-116 cells can serve as a model for investigating the role of high confidence DMRs in colon cancer. As observed previously, we also observed HCT-116 cells to be sensitive to the demethylating agent decitabine [31]. Decitabine inhibited clonogenicity (**Figure 2**) and demethylated genomic DNA (**Figure 3**). By bisulfite PCR, we observed DMRs previously identified in patient colon...
cancer cells to be consistently hypermethylated in HCT-116 cells. Furthermore, we achieved single-nucleotide resolution of CpG methylation at these DMRs. We were able to confirm previously identified CpG sites as well as identify new ones (Figure 4).

The HCT-116 model of DMR methylation has potential to shed light on the role of DMRs in colon cancer pathogenesis. Moving forward, it will be important to determine if (a) DMRs reduce nearby gene expression, (b) if DMR demethylation increases gene expression, and (c) if restoring expression of methylation-silenced genes affects HCT-116 growth or survival. In future, we plan to increase resolution at DMRs relevant to HCT-116 cell growth by sequencing across the entire DMR and by testing additional DMRs. The most updated data can be viewed using the UCSC genome browser public session found here: https://bit.ly/UCSC-DMR-methylation. The original data presented in this manuscript will be maintained as described in the data availability section.3,41.
Future work is needed to address the question if specific gene products suppressed by hypermethylation play a role in tumor growth. Nine frequently hypermethylated genes have been observed to slow cancer cell growth when heterologously expressed. Two additional frequently hypermethylated genes have been observed to inhibit cancer cell colony formation when heterologously expressed. Moving forward, we seek to heterologously express gene products suppressed by hypermethylation to test if such expression affects tumor cell growth and colony formation.

Data availability

Underlying data

Zenodo: williamhconrad/HCT116-DMR-bisulfite-PCR: bisulfite PCR repository under CC0 license. http://doi.org/10.5281/zenodo.3948439^28

This project contains the following underlying data:

- “Underlying data for Figure 2 - clonogenic survival.xlsx” (a spreadsheet containing the clonogenic survival data depicted in figure 2)
- “Underlying data for Figure 4b - UCSC methylation tracks for DMSO.bed” (a spreadsheet (tab-delimited bed format) containing the percent methylation data for DMSO treated cells presented in figure 4b)
- “Underlying data for Figure 4b - UCSC methylation tracks for decitabine.bed” (a spreadsheet (tab-delimited bed format) containing the percent methylation data for decitabine treated cells presented in figure 4b)
- “Underlying data for Figure 4c and d raw methylation quantification.xlsx” (a spreadsheet containing the raw methylation data depicted in figures 4c and d)
- “Underlying data for table 4 DMR TF dataset-FINAL.xlsx” (a spreadsheet containing the transcription factor binding data for DMRs evaluated in table 4)
- fig 3 raw hpaii digest image 300 dpi.tif (Raw gel image for Figure 3)

Zenodo: williamhconrad/HCT116-decitabine-hub: Decitabine Hub repository under CC0 license. http://doi.org/10.5281/zenodo.3946753^29

This project contains the following underlying data that are used to build the UCSC genome browser public hub found at https://bit.ly/UCSC-DMR-methylation:

- “description.html” (An html file that describes the Tracks in this repository. This html file is used by UCSC genome browser to build a description for the public hub)
- “description.fld” (A folder with formatting for the file “description.html”)
- “genomes.txt” (A file used by UCSC genome browser to select the correct genome to annotate the methylation data)
- “hub.txt” (A file used by UCSC genome browser to find the DNA methylation tracks)
- “BMB322L-pctMethyl-DMSO-20200602.bed”, “BMB322L-pctMethyl-DMSO-20200602.bed”, and “BMB322L-pctMethyl-DMSO-20200602.bed” (identical spreadsheets in three formats containing the percent methylation data for DMSO treated cells presented in figure 4b for use by UCSC genome browser. The “.bb” and “bigBed” files are in bigbed format, the “.bed” file is in bed format.)
- “BMB322L-pctMethyl-Decitabine-20200602.bed”, “BMB322L-pctMethyl-Decitabine-20200602.bed”, and “BMB322L-pctMethyl-Decitabine-20200602.bed” (identical spreadsheets in three formats containing the percent methylation data for Decitabine treated cells presented in figure 4b for use by UCSC genome browser. The “.bb” and “bigBed” files are in bigbed format, the “.bed” file is in bed format.)

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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In this study, Sandler et al. investigated methylation status of six genes (DKK3, EN1, MiR34b, SDC2, SPG20, and TLX1) in colorectal cancer. By using the HCT116 CRC cell line as a model for CRC, the authors show that all six analyzed genes are methylated. Using bioinformatics approaches, they show that methylation inhibited transcriptional enhancer binding. Treatment of HCT116 cells with the demethylation compound Decitabine (5-Aza-2'-desoxycytidin) decreased the methylation of all genes. Finally, Decitabine treatment inhibited the clonogenic survival of HCT116 cells.

The six genes were selected based on a previous genome-wide study that identified 2648 differentially methylated regions in CRC. However, Sandler et al. did not describe what was the criteria to select the six analyzed genes.

By performing bisulfite sequencing they provide methylation status of the six genes at single nucleotide resolution. However, Cancer Cell Line Encyclopedia (CCLE) provides RRBS genome-wide methylation analyzes of 59 CRC cell lines. Therefore, the methylation status of the six genes can be also extracted from CCLE data.

The bioinformatics analyzes suggest that methylation inhibits transcriptional enhancer binding to the six genes. However, these findings needs to be experimentally validated before drawing any conclusions.

It has been shown in numerous studies that Decitabine inhibits the viability and clonogenic survival of various cancer cell lines, including HCT116. Therefore, this part of the study does not provide any new findings.

Altogether, I believe that the novelty and the study design are not sufficient for indexing.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Cancer research

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 27 August 2020

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Review comments:
This study investigates DNA methylation levels of DMRs in a single-nucleotide resolution using bisulfite sequencing. The selected DMRs are at the promoter regions of six genes based on published data from others. HCT116, a commonly used cell line in CRC cancer served as an acceptable model in this study. Decitabine (5-aza-2'-deoxycytidine) treatment in HCT116 significantly reduced CpG methylation at the DMRs, as well as clonogenic survival after a 5-day treatment, all consistent with published data. Overall, while the study is thoughtfully put together,
the scope is quite limited, but it can make a certain level of contribution to CRC research. The number of studies using HCT116 and decitabine is very large, and the authors would benefit from additional literature searches to put their data in to the context of what has been done in this area, which is extensive. Thus a key question with regard to publish/not publish, is whether the manuscript adds to what is already known. It is possible that for these loci, the data may be novel, but then again many studies have used HCT116 and decitabine with various omics methods (e.g. 450k array) so it would be worth checking if the sites examined here overlap with those in omics analyses to help determine novelty of what is shown here.

The manuscript is well written. However, there are a number of issues with the methods and analysis that need to be clarified/addressed or repeated. Adding a piece of extra bioinformatic data and RT-PCR results are also necessary to make clarify the study conclusions. These issues, and those mentioned above, prevent me to endorse its acceptance at the present stage. Below are more specific comments by section.

Points to address:
Replacing the most recently updated literature about CRC epidemiology, biology and epigenetics, and more accuracy of descriptions of what this literature has shown to improve the relevance of the study. As mentioned above there have probably been 100's of papers looking at methylation in CRC and using HCT116 cells as a model. Use of TCGA CRC data, which may or may not encompass the CpGs examined (they used the 450k array) is also strongly recommended.

The in silico analysis of transcription factor binding in relation to DNA methylation is clever, however the only really way to test this effect formally is to do experiments like EMSA with methylated/unmethylated problems and ChIP on methylated or unmethylated cells. So the outcome of the in silico analysis should be highly qualified.

The authors selected six DMRs proximal to DKK3, EN1, mir34b, SDC2, SPG20, and TLX1 genes from 2648 available DMRs. As all these DMRs were identified in 24 tumors and matched normal colon samples in a previous publication, due to the heterogeneity of CRC, it is hard to conclude that the methylation status behaves similarly in CRC cancer cell lines. There is a publicly available database (CCLE-https://portals.broadinstitute.org/ccle) in which DNA methylation data (RRBS) and RNA-seq results can be extracted for certain cancer cell lines including HCT116 and other 59 CRC cell lines. Integrating this piece of data will help to validate choice of HCT116 cells as a viable model for primary CRC and why these 6 DMRs were picked up for validation in this study.

Rationale for choosing these DMRs needs to be improved.
RT-PCR for gene expression is necessary to show the inverse relationship between DNA methylation and gene expression. It will also support the results found in the transcriptional enhancer binding analysis.

Transcriptional enhancer binding analysis: DMR Methylation was arbitrarily recorded and put into different methylation groups. Can cut off values be provided to support regrouping?
Clonogenic survival assay: It is better to seed cells after drug treatments to remove the confounding effect caused by the toxicity of decitabine. It is hard to explain why there is no colony formed even using 0.25µM of decitabine. The clonogenic survival assay should be redone to remove the confounding effect. But again, this assay has already been done by others. What would add true novelty here would be to CRISPR target a single hypermethylated gene, then do a
clonogenic assay.

Global demethylation analysis: Recommend to use MspI-HpaII pair rather than HpaII itself. MspI is an isoschizomer of HpaII which cleaves both unmethylated and methylated HpaII sites, but HpaII is unable to cleave the site when the inner CG dyad is fully- or hemi- methylated. Similar digestion patterns will be observed when unmethylated DNA was treated with MspI and HpaII, while different patterns will show up for methylated DNA.

Bisulfite PCR and sequencing: In most publications, bisulfite PCR and sequencing clones is preferred. The authors should detail the accuracy of their method using the relative peak height of cytosine and uracil at a given CpG site to calculate percent methylation. It seems like standards would be needed for this method to be quantitative.

Table 2 and 3 can either be supplementary tables or be described in the context of the methods section. It is not necessary to list as tables in the main section.

Results P5: The author stated that “In mammals, cytosine methylation inversely correlates with gene expression”. It seems true mostly in cases of DNA methylation in the promoter regions, but not in gene body. Please qualify.

P5: Methylation inversely correlates with transcriptional enhancer binding: Is there specific supporting data for HCT116?
Table 5: Chi square calculation is not correct (the expected values) for table 4 in the underlying data.

Figure 2: There is no colony formation when HCT 116 is treated by 0.25µM and 1 µM decitabine. It might be caused by non-specific drug toxicity. Repeating this experiment by plating cells after drug treatments is strongly recommended.

Figure 3: What causes difference of bands in column 1 and 3? It is better to add MspI digestion beside HpaII.

Figure 4b: Why are the length of the sequenced regions different for DMSO and decitabine? There are fewer CpGs sequenced in the decitabine treatment group than in the DMSO group. Figure 4c and 4d: As different numbers of CpGs are included for the 2 treatment groups (DMSO and decitabine), comparison of the methylation percentage between groups is biased. Because this is a main result in this study, the authors need to deal with this problem.

Discussion first sentence on P.6 in this section (“From our results, we conclude that HCT-116 cells can serve as a model ...”): the conclusion overreaches based on the data collected. Six DMRs cannot represent the whole picture of DNA methylation in primary CRC.

Even though the authors planned to test gene expression near the selected DMRs in the future, it is recommended to add the gene expression data in the current study to make a better story.

Is the work clearly and accurately presented and does it cite the current literature? Partly
Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: epigenetics, DNA methylation, cancer

We confirm that we have read this submission and believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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