Abstract:

Chromatin regulation plays a role in establishing and maintaining cell identity, and is generally highly dynamic between stem and differentiated cells. The intestinal epithelium is a monolayer of cells that is replaced every 3-5 days, and is thus a rapidly self-renewing and differentiating tissue. It is not currently understood how the integrity of the intestinal stem cell (ISC) genome is maintained or how the identity of a differentiated cell is dictated. Vital to chromatin regulation and gene expression are epigenetic modifications, such as methylation. The addition of methyl groups to the nucleotide cytosine silences genes, while demethylation allows gene expression. Demethylation pathways produce the epigenetic mark 5-hydroxymethylcytosine (5-hmC) as an intermediate. The conversion of 5-methylcytosine (5mC) to 5-hmC is accomplished by ten-eleven translocation (TET) enzymes, which require co-factors, such as the micronutrient alpha-ketoglutarate (a key intermediate in the Krebs cycle). Assessing the global 5-hmC abundance among distinct cellular populations will elucidate this epigenetic mark's role in determination of cell fate. To investigate this aim, murine intestinal organoid cultures were exposed to varying dosages of dimethyl 2-oxoglutarate, a cell permeable form of alpha-ketoglutarate, in order to modulate TET activity and thus 5-hmC abundance. Through dot blot analysis of 5-hmC abundance, the ability to modulate the epigenetic mark was assessed. Results indicate that exposure of intestinal organoids to dimethyl 2-oxoglutarate leads to phenotypic differences compared to controls, but these differences cannot be attributed to 5-hmC abundance. Understanding the differences in 5-hmC across cellular populations will provide insight into the role of epigenetic modifications in regulating gene expression. Furthermore, the impact of micronutrients on the genome emphasizes the significance of cellular environment for cellular identity.

Introduction:

Each cell in an individual holds the same genome, and yet over one hundred different cell types arise from this identical sequence of adenines, cytosines, thymines, and guanines. The human genome is comparable to a book: the nucleotides, letters, are ordered to form genes, sentences, combinations of which come together to create the proteins that fill and power the many diverse cells of the body, separate chapters of one complete book. In order for proper development to occur, every cell must correctly express the genes required to carry out its specific physiological function. How is this direction of cell fate regulated? Evidence shows that methylation and demethylation are involved.

DNA methylation is a widespread epigenetic modification; the human genome is >70% methylated at any given time[1]. Methylated cytosine, or 5-methylcytosine (5mC), plays a critical role in gene silencing, genomic imprinting, X chromosome inactivation, as well as the stability of genomic DNA[2]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), three of which are enzymatically active (DNMT1, DNMT3A, and DNMT3B)[3]. Errors in DNA methylation can cause embryonic lethality and cancer[4]. Cytosine can be demethylated by recently discovered mechanisms[5], presenting 5-hydroxymethylcytosine (5-hmC). As an intermediate of active demethylation, 5-hmC is involved in the pluripotency of stem cells, development, and disease[6].

The conversion of 5mC to 5-hmC is accomplished by ten-eleven translocation (TET) enzymes, which are named after the rare (t(10;11)(q22;q23)) translocation commonly seen in acute myeloid leukemia and lymphocytic leukemia[7]. There are three TET proteins in the family, TET1, TET2, and TET3, all of which are dependent upon Fe^{2+} and 2-oxoglutarate[8]. 2-

oxoglutarate is an intermediate in the Krebs cycle, and the ratio of 2-oxoglutarate to succinate has been shown to impact TET-dependent demethylation[9].

Therefore, experimental dosing of organoids with cell permeable forms of 2-oxoglutarate and succinate can be used to enhance or inhibit the conversion of 5mC to 5-hmC. 5-hmC abundance has been seen to vary during development and differentiation[10]. Manipulating the expression of 5-hmC and understanding the impact that its presence has is the first step in understanding its role in the regulation of cell fate.

Our lab has previously shown, utilizing a transgenic *Sox9*^{EGFP} mouse, that populations of cells expressing different levels of the transcription factor *Sox9* correlate to distinct phenotypes[11] (Figure 1B). *Sox9*^{High} marks reserve intestinal stem cells (rISCs), *Sox9*^{Low} marks active intestinal stem cells (aISCs), *Sox9*^{SubLow} marks progenitor cells, and *Sox9*^{Negative} marks differentiated cell types such as Paneth cells, Goblet cells, and absorptive enterocytes[2]. The ability to isolate distinct populations of cells (Figure 1C) from one reporter mouse is advantageous because it allows for the analysis of the entire intestinal epithelial differentiation landscape (Figure 1A), from ISCs to post-mitotic cells, enabling us to better comprehend how genetic/epigenetic changes regulate cell fate. Preliminary sequencing data indicates that 5-hmC is present in all *Sox9*^{EGFP} populations. This may in part explain the mechanism of differential expression of cellular populations in the intestine.

To address this research question our lab has been utilizing advanced sequencing techniques to map 5-hmC across the landscape of intestinal differentiation. Preliminary 5-hmC mapping results indicate differential enrichment of 5-hmC across the genome of *Sox9* populations (Unpublished data, n=3)(Figure 1E). This analysis was accomplished beginning with fluorescence activated cell sorting (FACS) of intestinal epithelium from *Sox9*^{EGFP} reporter mice,

and the isolation of four distinct cell lineages. 5-hmC was then assessed at genes of interest in these populations using a pull-down technique (hmC-Seal) followed by next-generation sequencing (NGS).

In order to assess the genomic distribution of 5-hmC large-scale mapping has been accomplished through multiple different methods. The most "straightforward"[12] method is 5-hmC immunoprecipitation, which utilizes antibodies; this method is expensive, and there have been notable variations in the results obtained from different experiments[13]. CMS immunoprecipitation involves treatment with sodium bisulphite, which converts 5-hmC to cytosine methylene sulphonate (CMS); this method recovers DNA regions containing 5-hmC with high specificity and low background, but it is prohibitively expensive. I chose to optimize the hmC-Seal pull-down because it provides an advantageous balance of affordability and accuracy.

To assess the relationship between 5-hmC and transcriptional regulation, I developed primary murine organoid cultures and treated them with the TET cofactor and cell permeable 5-hmC agonist dimethyl 2-oxoglutarate in order to assess how 5-hmC levels impact gene expression. Organoids are self-organizing three-dimensional structures derived from primary tissue stem cells that exhibit similar functionality as the tissue of origin[14]. Organoids were analyzed phenotypically every two days, and their Paneth cells were counted. Successful modulation of 5-hmC abundance was validated via dot blot after organoids were lysed.

In order to test whether dot blot assays were a viable method of assessing global 5-hmC expression, a preliminary dot blot was carried out on UEA positive and negative FACS-sorted cellular populations. UEA is an absorptive enterocyte marker which binds exclusively and specifically to intestinal epithelial cell brush borders[15].

The conversion of 5mC to 5-hmC is an important mechanism to study, as 5-hmC plays a role in the regulation of gene expression during cellular differentiation, as well as the maintenance of pluripotency[16]. Thinking of the genome as a book, we propose that epigenetic marks, such as 5-hydroxymethylcytosine, act as highlighters, indicating which specific genes the cell should express. Results indicate that dimethyl 2-oxoglutarate impacts organoid phenotype, but the causality of this relationship has yet to be attributed to 5-hmC abundance.

Methods:

Mapping 5-hmC across the landscape of intestinal differentiation. Initial 5-hmC mapping was accomplished via hmC-Seal pull-down. The process begins with the addition of azide-modified glucose to the hydroxyl group of 5-hmC via T4 β-glucosyltransferase. Another reaction couples this azide-modified glucose to a biotin molecule (Figure 1D). Streptavidin coated beads bind to this biotin to allow pull-down. Through this technique, DNA enriched for 5-hmC can be gathered for downstream analysis by NGS. I optimized this hmC-Seal protocol, focusing specifically on testing T4 β-glucosyltransferase concentrations in order to increase 5-hmC enrichment efficiency. Subsequent qPCR analysis was used to validate 5-hmC-seq at selected regions of the genome where 5-hmC signal is detected (Figure 3).

Furthermore, to enhance the understanding of 5-hmC across the landscape of intestinal differentiation, cells sorted via FACS based on *Sox9* expression representing the four distinct cellular populations were analyzed via qPCR for TET and DNMT activity, which are crucial for 5-hmC presence. DNMT's deposit the methyl group on the cytosine, and TETs convert that methyl group to a hydroxymethyl group, creating 5-hmC. In order to accomplish this, FACS-sorted cells were lysed, purified RNA was synthesized via the Ambion™ RNAqueous® Micro

RNA isolation kit. Subsequently, cDNA was synthesized from the isolated RNA, 1 microliter of which is added into a qPCR plate that contains a 9 microliter master-mix of SsoAdvanced™ Universal Probe Supermix (5 microliters), molecular grade water (3.5 microliters), and the TaqMan Probes for the gene of interest (0.5 microliters).

Establishing the functional relationship between 5-hmC and transcriptional regulation. To assess the impact of 5-hmC on gene transcription *in vitro*, 5-hmC was modulated through exposure of primary murine organoids to cell-permeable forms of alpha-ketoglutarate, dimethyl 2-oxoglutarate. Intestinal organoid culture systems allow for *in vitro* assays that maintain normal epithelial differentiation and self-renewal. Organoids were established from wild type C57B1/6 mice. Three mice were sacrificed, their jejunums extracted, and the crypts (where the stem cells reside) isolated. These crypts were concentrated and placed into Cultrex, a three-dimensional matrix-gel that is liquid at 4 degrees Celsius but polymerizes at room temperature. This matrix was deposited in a bubble into a 96 well plate. Each well holds a 10 microliter bubble of Cultrex, which holds about 50 crypts initially, and is covered in 100 microliters of ENR growth media. ENR growth media contains 84.6% Advanced DMEM/F12, 0.9% Glutamax, 0.9% HEPES Buffer, 1.8% B27, 0.9% P/S, 0.9% N2, 10% Rspo1, 1,000x Noggin, 10,000x EGF, and 1,000x Y27 on the first day.

Organoids were allowed to develop for six days before dosing with dimethyl 2-oxoglutarate began. On the sixth day after plating, the organoids were exposed to various concentrations of either micronutrient (0, 1, 2, 5 and 10 mM) every 48 hours when the ENR growth media was changed. While exposing these organoids to dimethyl 2-oxoglutarate, I observed cell survival by counting organoid number per well on the microscope every 48 hours before dosing, and noted any phenotypic abnormalities. I counted the Paneth cells per organoid

bud for 20 buds per well while organoids were being dosed. Images (Figure 4A) were taken on an Olympus IX-81 with a 40X objective.

After the sixth day of dosing with dimethyl 2-oxoglutarate the organoids were ready to be processed. One third of the organoids were lysed for gDNA to be analyzed via dot blot, one third of the organoids were lysed for RNA to be analyzed by qPCR, and the remaining third were fixed and embedded to later be sectioned and stained via immunohistochemistry.

Results:

In order to investigate the presence of 5-hmC, the machinery for methylation and demethylation was also analyzed. Flow cytometry was utilized to sort primary murine cells into distinct Sox9 populations (Figure 1C). qPCR analysis of RNA transcript levels in FACS sorted cells indicates that TET proteins, the enzymes which convert 5mC to 5-hmC, are seen to be expressed at different levels throughout the distinct Sox9 populations of the small intestine (Figure 2). The family of active DNA methyltransferases, DNMT1, DNMT3A, and DNMT3B, involved in the methylation of cytosine, are also expressed at different levels throughout the distinct *Sox9* populations (Figure 2).

Organoids treated with varying doses of dimethyl 2-oxoglutarate exhibited noticeable phenotypic differences and altered survival times as compared to control organoids. Six days after initial dosing with dimethyl 2-oxoglutarate began it was visible that the organoids exposed to the 10 mM concentration of dimethyl 2-oxoglutarate exhibited different phenotypes (Figure 3A). In the absence of dimethyl 2-oxoglutarate (Figure 3A, Panel 1) a handful of dark and granular Paneth cells can easily be seen. As the concentration of dimethyl 2-oxoglutarate increases the Paneth cell count per bud decreases, and the organoid buds appear withered and

unhealthy. This visual phenomenon has been empirically represented in a graph of average Paneth cell count per bud over the course of the experiment (Figure 3B). 140 buds were counted for 0 and 1 mM each, 141 buds were counted for 2 mM, and 134 buds were counted for the 5 mM concentration. This data was gathered from six technical replicates of organoids derived from three biological replicates. No data was collected for 10 mM wells because the organoids began to die.

Increasing levels of dimethyl 2-oxoglutarate decreases organoid survival. This data is represented as percentage of initial organoids per well (Figure 5) over the course of the experiment. This data was gathered from five biological replicates of organoids from two separate mice.

Dot blot validity was assessed by measuring the expression of 5-hmC for serial dilutions and comparing it to an exponential curve, decreasing by a factor of 2 each dilution. 5-hmC expression followed an exponential trend down the blot (Figure 7), indicating proper serial dilution of samples and loading of gDNA onto the nitrocellulose membrane. This dot blot indicated that UEA positive and UEA negative populations express different amounts of 5-hmC (Figure 6).

Dot blots analysis of primary murine organoids exposed to dimethyl 2-oxoglutarate are ambiguous at the moment (Figure 8). Although an initial dot blot indicated that increased concentration of dimethyl 2-oxoglutarate correlated with increased global 5-hmC expression (Figure 8A), Methylene Blue analysis of the nitrocellulose blot revealed unequal gDNA deposition, and therefore improper loading. The second dot blot indicated that increased concentration of dimethyl 2-oxoglutarate had no impact on global 5-hmC expression (Figure 8C). Methylene Blue confirmed proper loading of this dot blot (Figure 8D).

Discussion:

The different levels of TET and DNA methyltransferase transcripts, as indicated by qPCR data (Figure 2), across the distinct cellular populations of the small intestine indicates that methylation and demethylation occur at different levels in these different cellular populations. Thus, as cells transition from stem to progenitor, and further from progenitor to differentiated cells, methylation profiles must change. Therefore, 5-hmC, as a mark of active demethylation pathways, is an attractive mark to analyze epigenetic regulation of cell fate and differentiation.

qPCR analysis of hmC-Seal pull-down products showed enrichment for 5-hmC over genes *Adh6a* and *Fabp2,* absorptive genes, compared to *Sox2* and *MyoD1* loci, which are involved in renewal and muscle development, respectively. It was confirmed that 5-hmC presence varies across cellular populations within the small intestine by carrying out a dot blot of gDNA of cells sorted into UEA positive and negative subpopulations (Figure 5). Cells that were sorted into a UEA-negative subpopulation expressed more global 5-hmC in their gDNA than cells from the same mouse sorted into a UEA-positive subpopulation.

Initially, dot blot analysis of organoid gDNA from this experiment implied that global 5-hmC was increased by exposure to higher concentrations of dimethyl 2-oxoglutarate (Figure 6A). However, a subsequent dot blot of organoid gDNA from a biological replicate indicated that 5-hmC was not expressed at different levels in organoids exposed to various concentrations of dimethyl 2-oxoglutarate (Figure 7A). Both of the dot blot results were confirmed by staining the nitrocellulose blots for whole gDNA presence with Methylene Blue. Methylene Blue staining confirmed that the first blot was incorrectly executed (unequal distribution of gDNA across blot, Figure 6B), and that the second blot was correctly done (equal distribution of gDNA across blot,

Figure 7B). However, if the second blot is accurate, this implies that 5-hmC presence is unaffected by increased exposure to dimethyl 2-oxoglutarate.

Although results have shown that 5-hmC is differentially expressed across different populations, the capability to modulate 5-hmC *in vivo* has not yet been validated via dot blot, but phenotypic results indicate a biological response to exposure to dimethyl 2-oxoglutarate. This may be attributable to the fact that the family of TET proteins contains three enzymes, all of which have slightly different functions, and dimethyl 2-oxoglutarate most likely impacts other aspects of cellular metabolism. Future experiments will analyze TET protein expression via qPCR to validate that TET mediated demethylation is increasing due to the dimethyl 2-oxlogutarate, in order to identify the mechanism of the phenotypic changes observed.

Acknowledgements:

I would like to thank my PI, mentor, and friend Dr. Adam Gracz for all of his help and guidance throughout this project.

Figures:

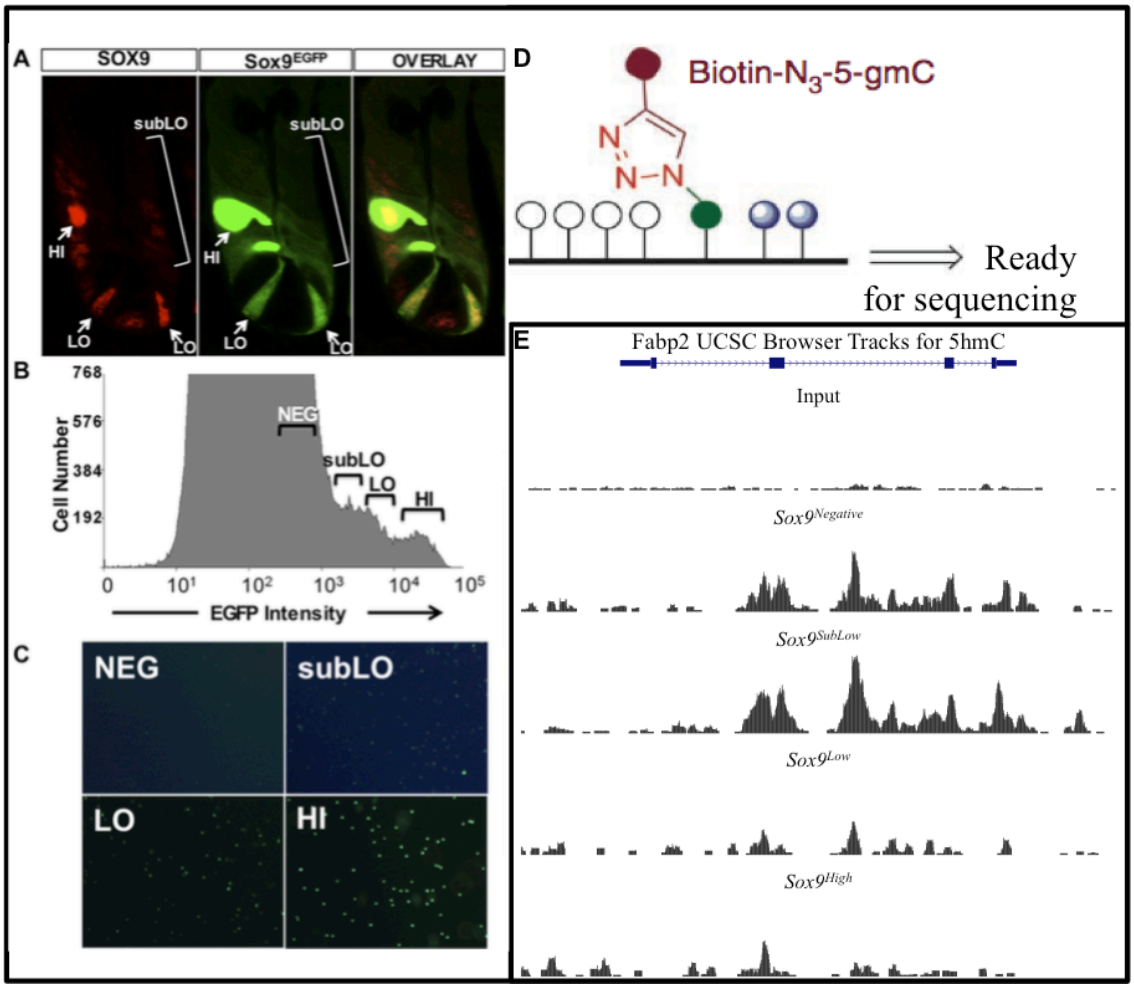


Figure 1: Population Identification and 5-hmC Enrichment. A) *Sox9*^{EGFP} is an accurate reporter in the intestinal crypt, because it recapitulates endogenous *Sox9* expression. **B)** Flow cytometric analysis illustrates distinct levels of *Sox9*^{EGFP} expression within intestinal epithelium. **C)** Single cells sorted by FACS into discrete populations. *Sox9*^{Neg} marks differentiated cells, *Sox9*^{SubLow} marks progenitor (transit-amplifying) cells, *Sox9*^{Low} marks active intestinal stem cells (aISCs), and *Sox9*^{High} marks reserve intestinal stem cells (rISCs). **D)** hmC-Seal pull-down requires several modifications to be made to 5-hmC positive DNA. An azide group is attached to the 5-hmC via chemically modified glucose. This azide group is labeled by a biotin moiety using click chemistry. Paramagnetic streptavidin beads are used to capture labeled DNA, which can then be sequenced. **E)** 5-hmC is enriched over *Fabp2* (fatty acid-binding protein 2) gene region, specifically in *Sox9*^{Negataive} and *Sox9*^{SubLow} populations. (Adapted from Gracz et al, 2010, Song et al, 2011, and unpublished data)

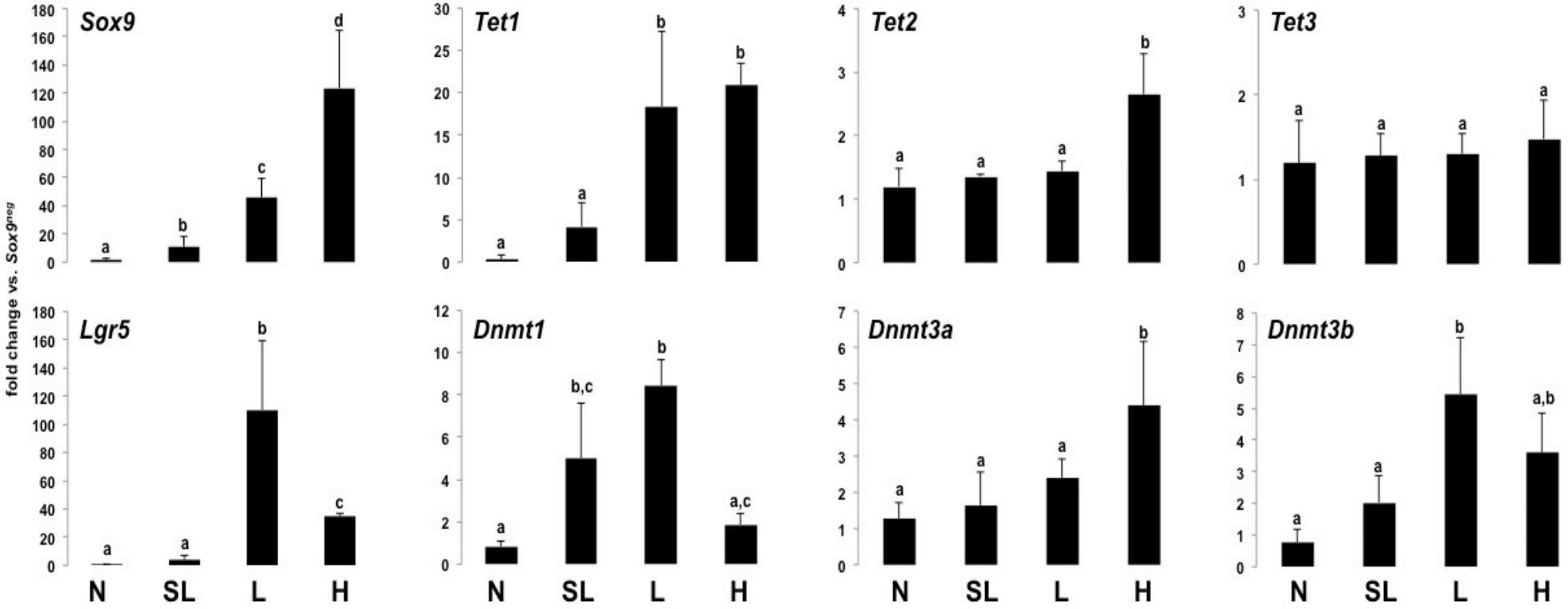


Figure 2: qPCR Analysis of Key Proteins. qPCR analysis of cells FACS-sorted by *Sox9* expression into the defined populations (N=Negative, SL=Sublow, L=Low, H=High) of the small intestine (as shown in Figure 1). qPCR analysis of *Sox9* confirmed that cells were sorted accurately, and qPCR analysis of *Lgr5*, a stem cell marker, confirms the distinct cellular populations are in different states of "stemness". RNA expression of the TET family of proteins as well as the DNMT family of DNA methyltransferases indicates different expression across distinct cellular populations. (n=3 biological replicates; different letters indicate statistically significant differences, $p<0.05$)

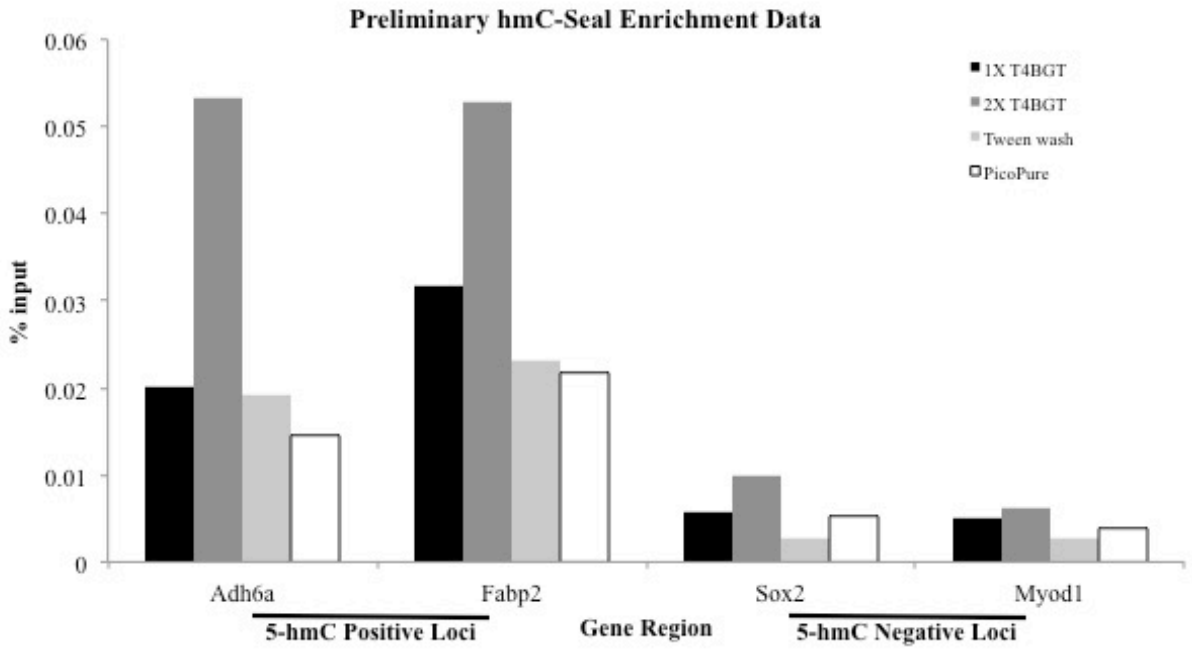
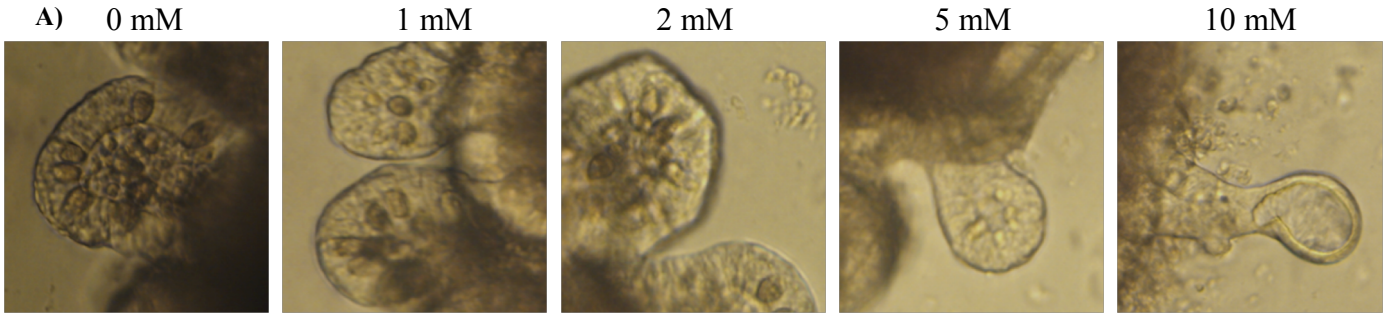


Figure 3: hmC-Seal Enriches for 5-hmC Positive Regions. hmC-Seal-qPCR indicates that *Adh6a* and *Fabp2* loci are enriched for 5-hmC, while *Sox2* and *Myod1* loci are not. *Sox2* and *Myod1* signal is considered background noise. Optimized protocol, which utilizes 2X T4BGT, is currently providing a 6.57-fold enrichment of signal over noise.



B)

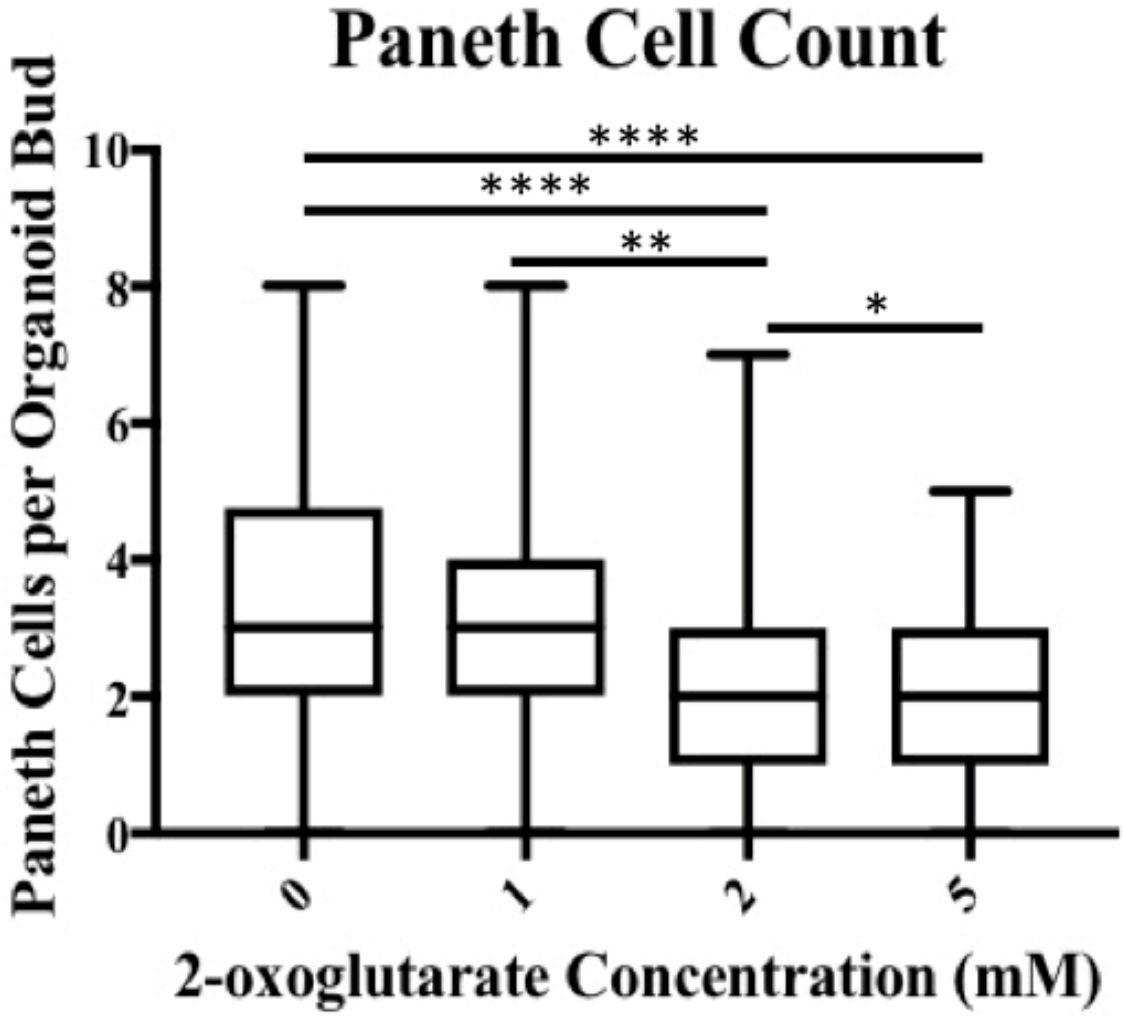


Figure 4: Analysis of Paneth Cells (darker, granular cells) in buds of organoids. A) Organoids were exposed to different dosages of 2-oxoglutarate, 0 mM through 10 mM. Images taken 6 days after dosing, 12 days after plating.

B) Average observed Paneth cell count per organoid bud. Mice, n=3. Technical replicates, n=6. Buds counted, n=140, 140, 141, 134 for 0, 1, 2, 5 mM 2-oxoglutarate dosages, respectively. Bars represent standard deviation. No data is presented for 10 mM because organoids did not express Paneth cells.

Statistical significance: 0 to 1 – 0.2478 (NS), 0 to 2 - <0.0001 (****), 0 to 5 - <0.0001 (****), 1 to 2 – 0.0073 (**), 2 to 5 – 0.0316 (*)

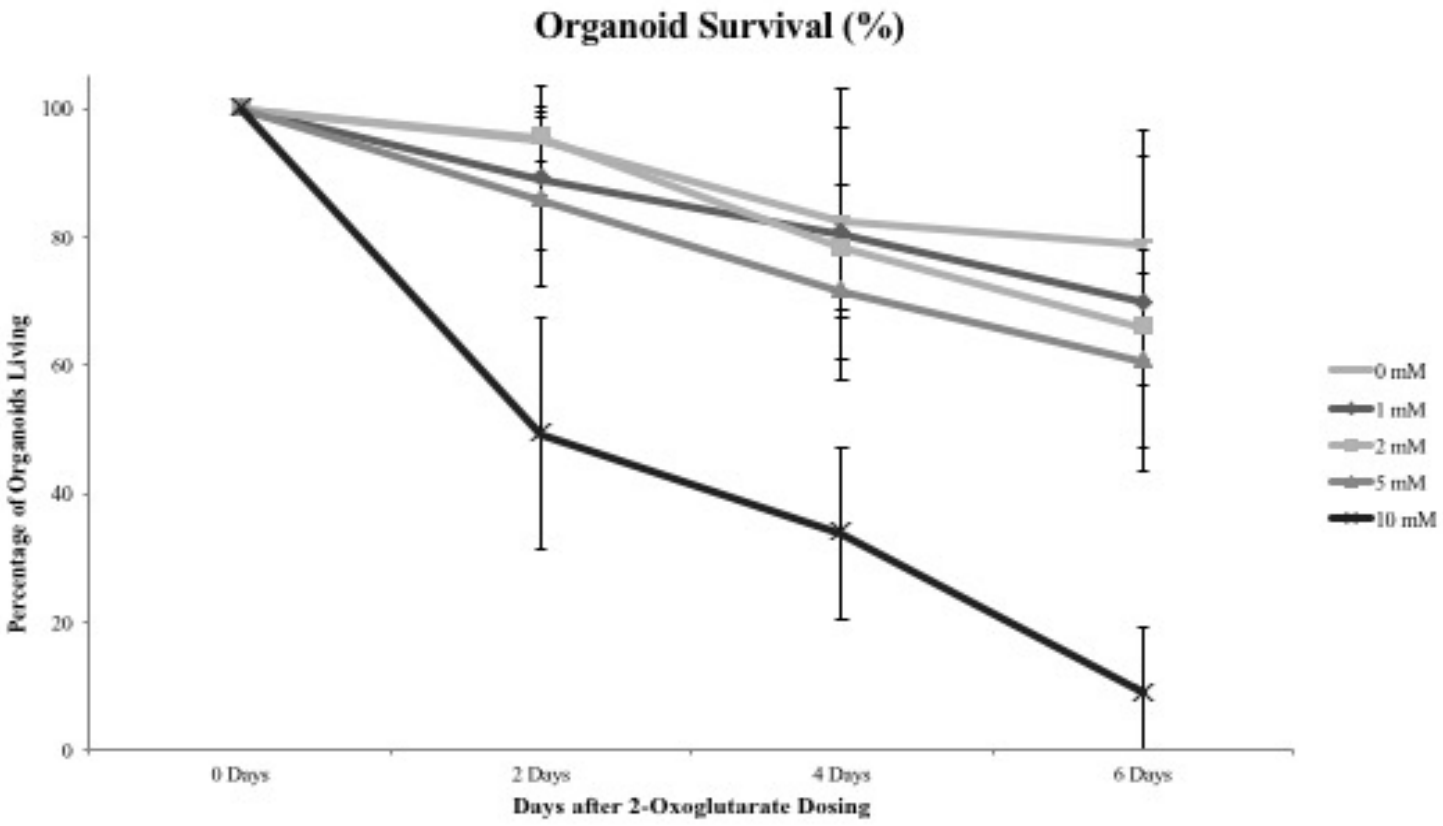


Figure 5: Organoid Survival: Organoid survival as a percentage of the total number of initially plated organoids per well. Each line represents a different concentration of 2-oxoglutarate.

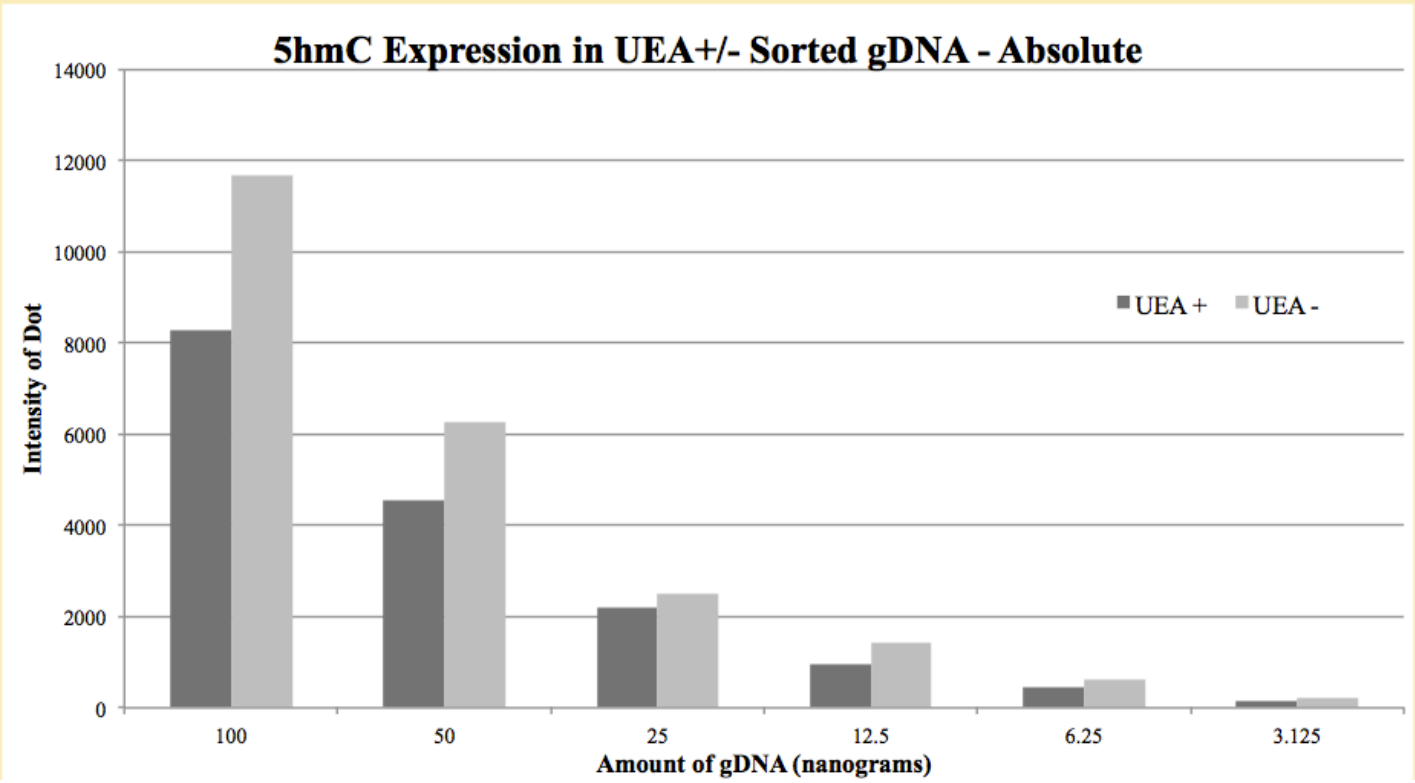


Figure 6: 5-hmC expression in UEA +/- populations: UEA positive and negative sorted cells were analyzed via dot blot for global 5-hmC presence. The y-value represents the intensity of the fluorescence of a HRP secondary antibody specific to the 5-hmC primary antibody, as quantified by ImageJ software.

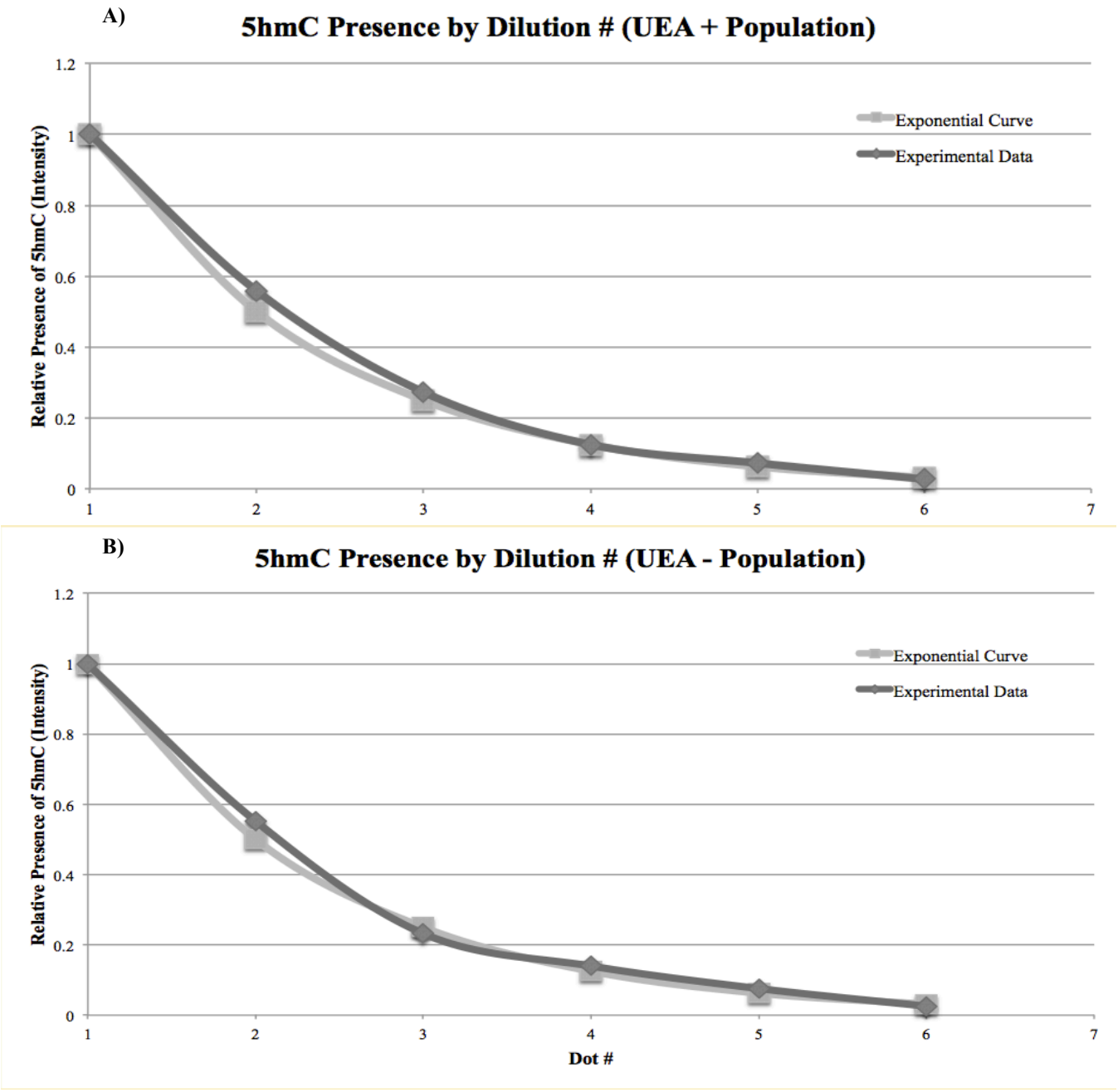


Figure 7: UEA Dot Blot Validation. Whole gDNA sorted into UEA positive **(A)** and UEA negative **(B)** samples. Validation that gDNA was serially diluted by factors of two correctly. 5hmC expression decreases along exponential curve (lighter gray line).

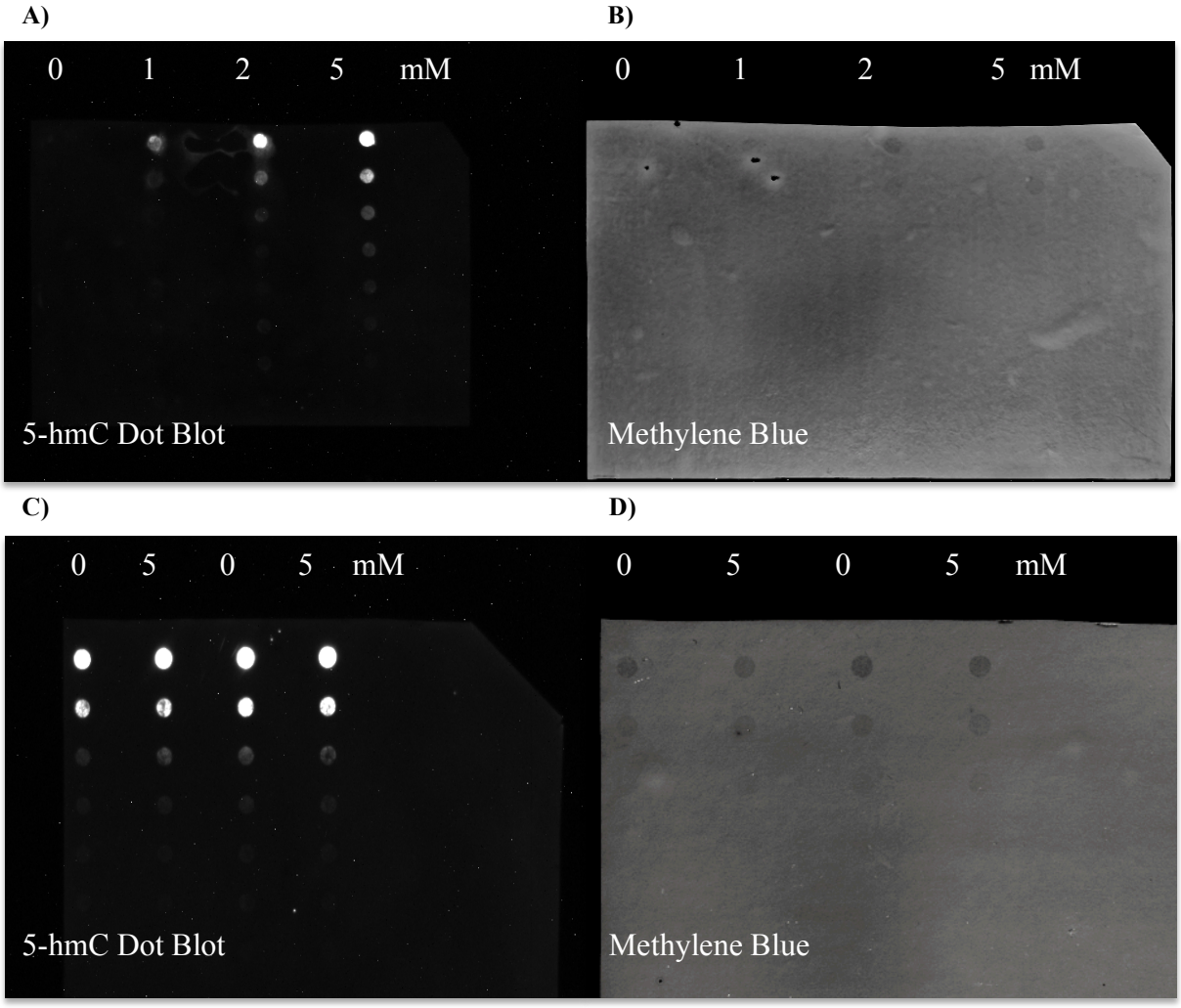


Figure 8: 5hmC Dot Blot. A) ECL Imaging of 5hmC signal from whole gDNA of organoids treated with 2-oxoglutarate. 2-oxoglutarate concentrations indicated above each column. **B)** Blots were also analyzed via Methylene Blue. **C)** ECL imaging of 5-hmC signal from whole gDNA of organoids treated with 2-oxoglutarate, different biological replicate than seen in panel A. **D)** Blots was analyzed via Methylene Blue to confirm gDNA presence in nitrocellulose membrane.

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