Mechanisms of Epigenetic Regulation of Gene Expression in Colorectal Cancer Cells

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

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Date David Mossman

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Table of Contents

Common Abbreviations

ChIP	Chromatin Immunoprecipitation
CpG	CpG dinucleotide sequence
CRC	Colorectal Cancer
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
5-aza-dC	5-aza-2-deoxycytidine
HPLC	High Performance Liquid Chromatography
H3	Histone H3
K	Lysine
MDS	Myelodysplastic Syndromes
me	methyl
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
TSA	Trichostatin A

Publications

A. Papers

1. Mossman, D. and Scott, R.J., *Epimutations, Inheritance And Causes of Aberrant DNA Methylation in Cancer.* Hereditary Cancer in Clinical Practice 2006; 4(2) pp. 75-80.

2. Reeves SG, Mossman D, Meldrum CJ, Kurzawski G, Suchy J, Lubinski J & Scott RJ., *The -149C>T SNP within the ΔDNMT3B gene, is not associated with early disease onset in hereditary non-polyposis colorectal cancer.* Cancer Lett 2008 June 28; 265(1):39-44.

3. Mossman D, Kim KT & Scott RJ, *Demethylation by 5-aza-2'-deoxycytidine in colorectal cancer cell lines targets genomic DNA whilst CpG island methylation persists.* BMC Cancer 2010, Jul 12;10:366.

4. Mossman D & Scott, RJ, *Long term transcriptional reactivation of epigenetically silenced genes in colorectal cancer cells requires DNA hypomethylation and histone acetylation. Accepted PLoS One,* July 2011.

5. Mossman D & Scott RJ, *Molecular responses of colorectal cancer cells to 5-aza-2'-deoxycytidine.* Submitted Mutagenesis and Carcinogenesis, July 2011.

B. Conference Proceedings

1. David Mossman & Rodney Scott, 'Prolonged transcriptional reactivation of epigenetically silenced genes requires hypomethylated CpG sites and histone acetylation', Epigenetics 2009, December 1-4, Melbourne, Australia.

2. David Mossman, Cliff J Meldrum & Rodney J Scott, 'Chromatin structure, DNA methylation and its relationship to gene expression'. Poster presentation at 'Stem Cells, Cancer and Aging', September 29-October 4 2008, Singapore, Singapore.

3. David Mossman, Cliff J Meldrum & Rodney J Scott, 'Chromatin structure, DNA methylation and its relationship to gene expression'. Poster presentation at 'Ten of the Best Research Showcase', September 26 2008, University of Newcastle, Callaghan, Australia.

4. David Mossman, Cliff J Meldrum & Rodney J Scott, 'Chromatin structure, DNA methylation and its relationship to gene expression'. Poster presentation at 'Hunter Medical Research Institute Conference on Translational Cancer Research', September 11-12 2008, Newcastle, Australia.

5. David Mossman & Rodney J Scott, ‘Cancer cells differ in their ability to perform DNA methylation which may be responsible for tumour development’. Poster presentation at the ‘Australian Society of Medical Research Meeting’, June 2 2008, Sydney, Australia.

6. David Mossman & Rodney J Scott. ‘Cancer cells differ in their ability to perform DNA methylation which may be responsible for tumour development. Poster presentation at ‘Epigenetics 2007’, November 4-7, 2007, Perth, Australia.

7. Nicholas Wong, John Heath, Elizabeth Agar, David Mossman, Rodney J Scott, Jeffery Craig, Richard Saffery and David Ashley, ‘Uncovering epigenetic changes in paediatric Acute Lymphoblastic Leukemia’, Gordon Research Conference, Cancer Genetics & Epigenetics, May 20-25, 2007, Il Ciocco, Lucca (Barga), Italy.

8. David Mossman and Rodney J. Scott, ‘Global DNA Methylation levels and response to treatment with a methyltransferase inhibitor’. Oral presentation at the ‘HMRI Cancer Research Program Meeting’, March 15-16, 2007, Newcastle, Australia.

9. David Mossman and Rodney J. Scott. ‘Global methylation analysis by High Performance Liquid Chromatography’. Oral presentation at ‘Graduate Students Day’, October 20, 2006, University of Newcastle, Australia.

10. David Mossman and Rodney J. Scott. ‘Global methylation analysis by High Performance Liquid Chromatography’. Poster presentation at the 11th International Congress of Human Genetics, August 6-10, 2006, Brisbane, Australia.

Abstract

The role of epigenetics in disease, particularly cancer, has been an emerging issue for the last decade. For disorders with a genetic component, it offers an alternative mechanism by which disease can initiate and progress. The involvement of epigenetic aberrations in malignancy is evident, with essentially all tumour types displaying variation from a normal epigenetic pattern. A great deal of knowledge can be gained by understanding the epigenetic processes within cells, and manipulation of these mechanisms may lead to more effective treatments and better outcome for individuals at risk of developing cancer.

Studies described in this thesis are aimed to better understand the processes of epigenetic control on gene expression and how they relate to colorectal cancer. Previous studies have identified a single nucleotide polymorphism in *DNMT3B* which is thought to alter the age of disease onset in individuals susceptible to colorectal cancer. The effect of this heritable genetic marker was examined in a larger population size and was found to have no effect on the age of disease onset. This study is described in Chapter 2, the results of which spawned an in-depth analysis of epigenetic change in colorectal cancer cell lines.

The process of DNA methylation was examined, whereby 5-aza-dC was used to demethylate DNA in cultured colorectal cancer cell lines. When the drug was removed from growth medium, inhibition of methyltransferases ceased and remethylation occurred. The resulting effect of gene expression was found to be dependent on initial DNA methylation patterns, and is described in Chapter 3. A

follow up study to this was undertaken to understand the interaction between DNA methylation and histone modifications. The differences between short term and long term reactivated genes after 5-aza-dC exposure depends on increased Histone H3 acetylation and localised hypomethylation. This study is described in Chapter 4.

An investigation of the gene expression profile changes in colorectal cancer cells after 5-aza-dC exposure is described in Chapter 5. A pattern of gene expression similar to healthy epithelial cells was not observed immediately, or ten days after 5-aza-dC treatment. A gene from the Protein Kinase C family was found to be commonly down-regulated with drug treatment. This may have pro-apoptotic effects however this may not be sufficient to induce cell death in these cells as 5-aza-dC is not an effective treatment in solid tumours.

The information described in this thesis will contribute to understanding the process of aberrant DNA methylation that is observed in tumour cells. Information of this nature may identify individuals who are genetically susceptible to the epigenetic inactivation of crucial genes. A complete understanding of the co-ordination of the regulatory proteins will enable more effective treatments against this aspect of malignancy.

Chapter 1

General Introduction

1.1 General Introduction

DNA is considered to be the blueprint of life. All of the information required for life is encoded in the DNA by sequences of four nucleotides: adenine, cytosine, guanine, and thymine. Approximately 20,000 clusters of nucleotides, called genes, are spread across the human chromosomes [1]. For protein coding genes, the order of the nucleotides within the genes governs the structure of proteins, the functional products of genes. Mutations in the DNA sequence can lead to changes in the corresponding protein, which in turn compromise the ability of the protein to serve its correct function. A wide range of conditions are the result of genetic mutations, and many diseases have been traced to a single genetic origin. However this is not the case for complex diseases which may involve several genetic and epigenetic factors.

As early as 1904 it was proposed by Wheeler & Johnson [2] that a modified cytosine may occur in DNA, and in 1925 Johnson & Coghill demonstrated that a methylated cytosine, 5-methylcytosine, was found naturally in the DNA of the tubercule bacillus [3]. Some years later in 1948, this finding was supported by the work of Hotchkiss who isolated 5-methylcytosine from calf thymus DNA [4]. Whilst it was identified that the function of 5-methylcytosine in bacterial cells was to protect the DNA from host defences, in 1975 Holliday & Pugh proposed that cytosine methylation in eukaryotes was involved with control of gene expression in development [5]. In the past three decades this idea has been thoroughly examined and it is well established that 5-methylcytosine plays a crucial role in the epigenetic control of gene expression.

The modern definition of epigenetics is the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence [6]. The most common epigenetic modification in humans is DNA methylation, where a methyl-group (CH_3) is attached to cytosine. In addition to this modification, there are various complex modifications present on the histone tails with which the DNA has a close association. It appears that the interplay between DNA methylation and histone modification is crucial to normal chromatin organisation and gene expression. Larger functional RNAs have been established and will be discussed shortly, but more recently, small RNA molecules termed microRNAs, have been identified that are capable of controlling gene expression, revealing yet another important level of epigenetic control [7].

1.2 - DNA methylation and Methyltransferases (DNMT)

The process of DNA methylation involves the enzymatic attachment of a methyl group (CH_3) to a cytosine residue (see figure 1). In eukaryotes, methylation occurs on cytosines which are immediately followed by a guanine residue (5'-CG-3'), in what is known as a CpG dinucleotide, where the 'p' refers to the phosphodiester (phosphate group) bond between the nucleotides. Non-CpG methylation is prevalent in human embryonic stem cells, however this disappears upon induction of differentiation [8]. The methylation of cytosine residues is catalysed by three DNA methyltransferases (DNMTs), namely DNMT1, DNMT3A, and DNMT3B, using *S*-adenosyl-L-methionine as a substrate [9]. DNMT1 is primarily a 'maintenance' methyltransferase, due to its preference for

a hemi-methylated DNA substrate [10]. DNMT1 has a unique N-terminal sequence (see figure 2) that allows it to localise directly to the replication foci during S-phase [11]. This is understood to restore the methylation patterns which is mirrored on the complementary DNA strand. DNMT3A and 3B are believed to be responsible for the *de novo* methylation of DNA.

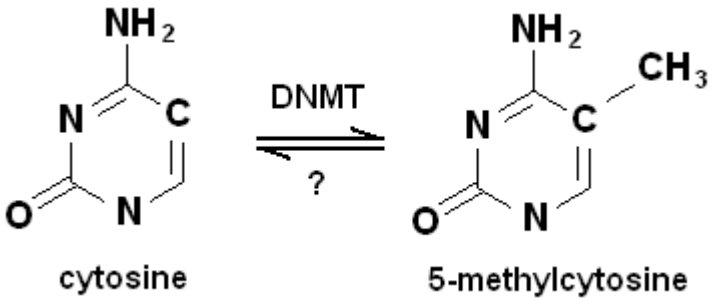

Figure 1 - The conversion of cytosine to 5-methyl-cytosine. DNMT enzymes catalyse the transfer of the methyl group to cytosine. It is not yet understood which protein(s) is responsible for the removal of the methyl groups.

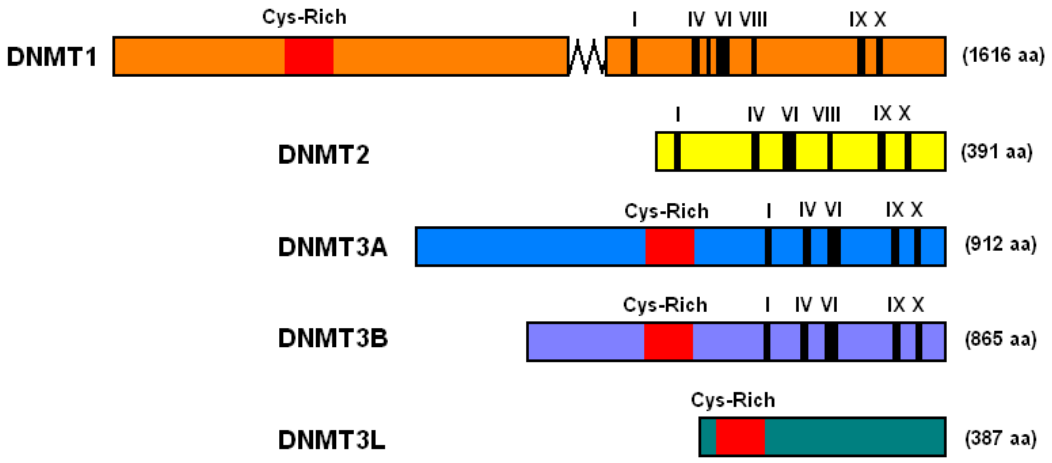

Figure 2 - Forms of Human Methyltransferase enzymes. DNMT1, DNMT3A and DNMT3B have the Cys-rich region and the conserved sequences, whilst the variants without methyltransferase activity lack these regions. Figure adapted from Bestor [12].

They exhibit a low level of expression in somatic cells which is in keeping with their designated *de novo* methylation abilities. However, despite DNMT1 being considered a maintenance enzyme, whilst 3A and 3B are *de novo* methyltransferases, there appears to be some overlap, and even co-operation, in how they function. Rhee *et al.* have demonstrated that double knock-out of *DNMT1* and *DNMT3B* results in a greatly reduced level of global DNA methylation and methylation of repeated sequences, as well as loss of aberrant methylation of the $p16^{INK4a}$ tumour suppressor gene [13]. This observation indicates DNMT3B is involved with maintenance of methylation. Similarly, DNMT1 has been shown to stimulate a five-fold increase in *de novo* methylation if both DNMT1 and DNMT3A are present [14]. These results suggest that the methyltransferases do have preferences for a particular DNA substrate but are likely to function in a broader manner than originally thought.

There are another two variants of DNA methyltransferase, but neither is regarded as having a substantial methyltransferase capacity. DNMT3L lacks critical domains that are present in the other functional methyltransferases, but appears to enhance the activity of *DNMT3A* [15] and *DNMT3B [16]*. In early development DNMT3L is thought to stimulate the activity of DNMT3A and is believed to be involved in the establishment of maternal genomic imprints [17] as will be discussed shortly. There is evidence to suggest that DNMT3L is able to stimulate the activity of both DNMT3A and DNMT3B both *in vitro* [18] and *in vivo* [19] via direct interaction.

Unlike DNMT3L, DNMT2 contains all the characteristic amino acid motifs found in the other DNMTs, albeit with a shorter N-terminus, yet no significant methyltransferase activity has been demonstrated. Hermann *et al*. [20] have shown that the enzyme has some residual activity on cytosines within a ttnCGga(g/a) consensus sequence, which might suggest the DNMT2 variant has a specialised purpose on particular DNA sequences. It has since been reported that DNMT2 is shown to be capable of methylating a cytosine within a tRNA molecule [21], and in doing so offering a suggestion as to why the conserved methyltransferase motifs are present in the DNMT2 protein.

Mutations within the *DNMT3B* gene have been described, and when both alleles are affected, lead to a disorder termed Immunodeficiency, Centromeric region instability and Facial anomalies (ICF) syndrome. Of the molecular changes reported, hypomethylation of regions on chromosome 1, 9 and 16 are the most consistent [22], along with hypomethylation of the X chromosome [23]. ICF is the only human disease to be associated with DNMT mutations, although mutations of *DNMT1* have been detected as rare events in colorectal cancers [24].

1.3 - Imprinted genes & X-inactivation

Bi-allelic DNA methylation plays a crucial role in the control of individual gene expression, as well as important regulatory functions associated with development. Within the genome are a group of genes that are expressed in a parental-specific manner, and these genes are termed imprinted genes [25]. Unlike other genes which are expressed from both alleles, normal imprinted genes are inactive on one allele, but are active on the other. One closely studied imprinted gene is the *IGF2* gene, or Insulin Like Growth Factor 2. The *IGF2* gene encodes a protein which is a growth factor involved with development and growth, and is expressed exclusively from the paternal allele, whilst the maternal allele is silenced [26]. Interestingly, another imprinted gene, *H19* is located approximately 100kb from *IGF2*, yet is expressed exclusively from the maternal allele and is silenced on the paternal allele [27]. Both genes are crucial for the normal placental development and foetal growth. Regulation of these genes relies upon the methylation of nearby differentially methylated regions and an enhancer region for correct expression [28, 29]. Bi-allelic expression of these genes (from both maternal and paternal alleles) is known as loss of imprinting and is commonly observed in cancer [30, 31], where epigenetic abnormalities are frequent. Whilst the imprinting process is important, it is not understood why some genes are controlled in this manner, and how the cell maintains the alleles in their respective opposite states.

X-inactivation is similar to imprinting, but occurs on a chromosomal level. It involves the suppression of an entire X chromosome, and is another example of epigenetically controlled gene expression. As females inherit two copies of the X

chromosome, one of the female X chromosomes is inactivated as a form of dosage compensation. Although the male genotype is characterised by one X and one Y chromosome, there are instances such as Klinefelters syndrome (karyotype: 47,XXY) where males possess an extra X chromosome. The extra genetic material results in developmental abnormalities, which do not eventuate in females due to X inactivation. Chromosomal inactivation arises from the expression of the *XIST* gene (X Inactive Specific Transcripts). This gene is located on the X chromosome and does not code for a protein, rather a functional RNA which initiates the exclusive silencing of the chromosome from which it was expressed [32]. During fertilisation, the paternal X chromosome is active but becomes suppressed with imprinted inactivation during the cleavage stage. Shortly afterwards this is reversed as observed by a loss of repressive histone modifications, and cells of the inner cell mass will give rise to the embryo where random X-inactivation occurs [33], and for each cell there is an equal probability of the maternal or paternal allele being inactivated [34]. However extreme skewing is sometimes observed, such that up to 90% of cells express the same X chromosome [35]. While healthy females may exhibit skewing, problems can arise when the active chromosome carries a mutated allele and skewing has been associated with recurrent abortion [36] and mental retardation [37].

1.4 - DNA packaging and Chromatin

The amount of DNA contained within each cell, if spread end to end, has been estimated to span a length of approximately two metres [38]. To enclose this much material into the nucleus of each cell requires a great deal of compression

and regulation. This is achieved by the DNA wrapping around a group of histone proteins to form a nucleosome, which represents the smallest unit of chromatin. Within each nucleosome, ~147bp of DNA is packaged around an octamer of two copies of each of the four core histone proteins, H2A, H2B, H3 and H4 [39]. The DNA-histone protein complex is then super-coiled around itself and with the assistance of a linker histone, H1, attached between the core particles, producing a stable and high level of compaction [40]. Figure 3 shows the model of DNA packaging best supported by data from biochemical and electron microscopy [41]. It is understood the nucleosomes act to inhibit gene expression [42], and the linker histone H1 may serve as a lock, holding the compacted chromatin together [43]. However, the structure of chromatin is dynamic and there are several factors which alter the conformation of the DNA, as discussed below.

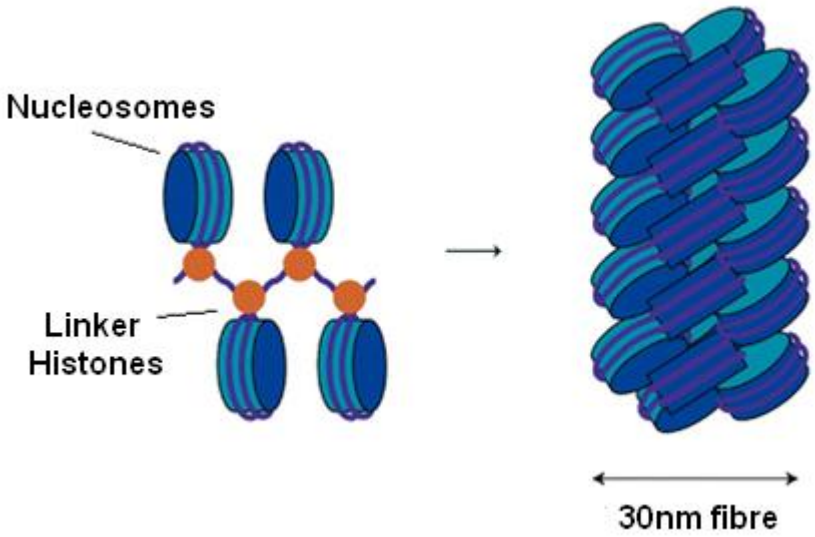

Figure 3 – The proposed zig-zag model of the structure of chromatin. The DNA is tightly associated with the histones and arranged in a manner that allows efficient compaction of the nucleosomes. Linker histones appear to hold the nucleosomes firmly in this position. This figure has been adapted from Khorasanizadeh [44].

1.5 - Epigenetic Regulation of Gene Expression

The conformation of the chromatin surrounding a gene plays a critical role in ensuring its correct expression. There are numerous alterations which occur both on the DNA and the corresponding chromatin which can alter the association between DNA, nucleosomes and other cellular proteins such as transcription factors or repressor proteins. DNA methylation is the predominant form of epigenetic modification of DNA, whilst many modifications to certain amino acids in the histone proteins are associated with either an active or a repressed state of gene expression.

1.5.1 - DNA Methylation

The methylation of DNA serves an important function in the control of gene expression, such as that seen in early development, but also forms the initial framework for other proteins to attach and confer stability to the chromosome. The bulk of non-coding DNA throughout the genome is methylated and compacted, assisting in keeping those regions in a transcriptionally inert state [45]. It is the co-ordination between DNA and several proteins that acts to impede transcription. It is well established that DNA methylation is repressive of gene transcription. The attraction of other protein factors to this methylated DNA actually blocks transcriptional machinery access to the DNA, and it has been argued that DNA methylation alone is not sufficient [46].

In 1989 a protein named Methyl-CpG binding protein 1, (MeCP-1), was identified which bound to a variety of methylated DNA sequences [47]. It was subsequently reported that transcription of methylated genes was allowed in the

absence of MeCP-1 binding, and transcription was repressed with its introduction [48]. Nowadays, it is commonly accepted that DNA methylation is the initial step which attracts the binding of proteins to co-operatively silence gene expression [49].

In addition to MeCP-1, a group of proteins belonging to a family of Methyl-CpG Binding Domain Proteins can also restrict DNA access to the transcriptional proteins. Members of this family are MBD1, MBD2, MBD3, MBD4 and MeCP2, which all have a common methyl-CpG binding domain. Each of these proteins, with the exception of MBD3, is able to bind to methylated DNA, and in the case of MeCP2, is capable of recognising a single symmetrically methylated CpG [50]. Interestingly MBD3 has been shown to bind only to un-methylated promoters [51] suggesting some slight variation in function between MBD3 and other family members. In addition to the binding of these proteins to repress transcription, they are also able to recruit other factors, such as nucleosome remodelling complex [52], which aid in converting the chromatin to an inactive form. With the exception of MBD4, all members of this family form complexes with Histone Deacetylase [53], a family of proteins which can alter the chromatin structure in a way that is repressive to gene transcription, as will be discussed shortly. Since the DNA methylation status of a gene strongly correlates with gene expression, it would suggest that methylated DNA does not frequently exist without the presence of methyl-CpG binding proteins, and detection of DNA methylation at a promoter region would indicate epigenetic inactivation at that locus.

1.5.2 - Acetylation

Within the N-terminal amino acid sequence of the core histone proteins are a subset of highly conserved lysine residues that can be subjected to acetylation [54]. The acetylation of histone proteins is a reversible process catalysed by two opposing families of enzymes. The Histone Acetyltransferases (HATs) catalyse the transfer of an acetyl group from acetyl coenzyme A to the lysine residue, whilst the Histone Deacetyltransferases (HDACs) catalyse the removal from the lysine, producing acetic acid as a by product, as shown in figure 4.

The main targets of acetylation are the lysine residues within histone H3 and H4, although H2A and H2B can also carry the modification [55]. Acetylation of lysine residues is associated with regions of active chromatin, whilst hypo-acetylated regions are found surrounding transcriptionally silent genes [56]. The introduction of an acetyl group neutralises the positive charge carried by the lysine, weakening the association between the nucleosome and DNA [57] and also the compaction of the chromatin [58]. The acetylation of core histone proteins has also been shown to directly assist binding of a transcription factor to model chromatin templates [59]. It would appear however, that DNA methylation is a more dominant regulator of gene expression than histone acetylation. Up-regulation of hyper-acetylated genes is observed when there is no promoter DNA methylation, and acetylation has little or no effect on a hypermethylated gene [60, 61].

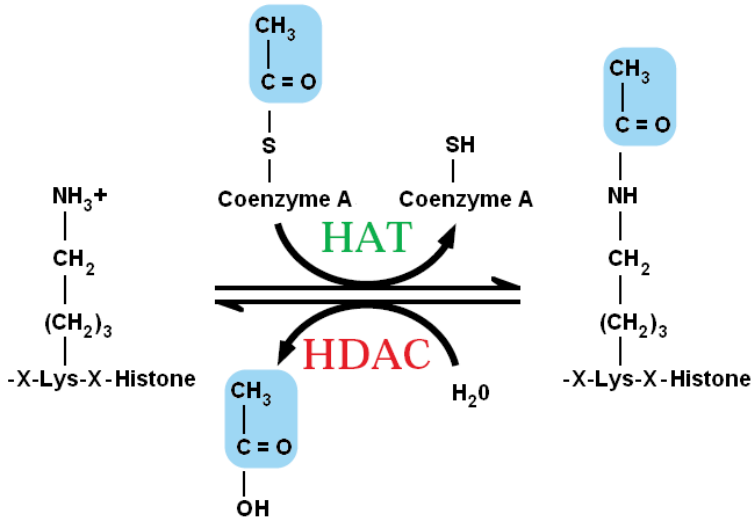

Figure 4 - The acetyltransferase and de-acetyltransferase reactions. Histone Acetyltransferase (HAT) catalyses the transfer of the acetyl group from acetyl coenzyme A. Histone Deacetyltransferase (HDAC) catalyses the removal of acetyl groups and in doing so converts water to acetic acid. Picture taken from Kuo & Allis [62].

1.5.3 - Histone Methylation

In addition to DNA methylation and histone acetylation, the core histone proteins can also be subject to methylation on arginine and lysine residues [63]. Similar to acetylation, histone methylation frequently affects histones H3 and H4, while H2A and H2B can also be modified [55]. A considerable proportion of the literature focuses on the methylation of lysine in histone H3, and is perhaps one of the more understood groups of histone modifications.

There are several lysine residues present in histone H3 protein which are able to be methylated, and the major modifications occur at H3K4, H3K9, and H3K27. In a fashion not unlike histone acetylation, the methylation is catalysed by Histone Methyltransferases (HMT's) [64], and demethylation can be catalysed by several proteins, including Lysine Specific Demethylase 1 enzyme [65].

Unlike DNA methylation, histone methylation has been associated with both active and inactive DNA regions, although this depends on the lysine(s) that are subjected to methylation. The methylation of Histone H3 Lysine 4 (H3K4me) is commonly found surrounding active genes, as is the methylation of H3K36 and H3K79, whilst methylation of H3K9 and H3K27 are associated with repression, particularly on the X-chromosome, as reviewed by Sims [66]. The fact that the methylation of two separate lysine residues can be only five amino acids apart and yet are distinctly opposite with regard to chromatin status provides strong evidence for the epigenetic code as a regulator of transcription. Lysine methylation can be in the form of mono- di- or tri- methylation, and while the significance of these is yet to be elucidated, specific modifications only appear under certain conditions. Histone H3 Lysine 4 tri-methylation (H3K4me3) is not usually present at a locus prior to transcriptional activation, whilst di-methylation (H3K4me2) may be present prior to activation [67].

1.5.4 - Other modifications

There are a range of other modifications that occur on the histone tails, including biotinylation, phosphorylation, ubiquitination and sumoylation. These modifications are less thoroughly studied than acetylation and methylation, and unlike the major modifications, appear to play specific roles not directly involved with gene expression. Biotinylation has been shown to repress transposable elements [68]; phosphorylation of linker histones appears to be involved with the timing of replication [69]; and ubiquitination is involved in several cellular processes such as the DNA damage response [70]. Histone sumoylation has been

linked with the repression of expression [71], but also receives little attention in the literature.

1.6 – DNA Methylation Patterns in Cancer and possible causes

Aberrant DNA methylation is one of the more frequent molecular changes in tumour cells [72] and typically involves the reversal of normal DNA methylation patterns (see Figure 5). Generally these changes involve genome wide hypomethylation, including that of oncogenes, loss of imprinting, and hypermethylation of tumour suppressor genes [73-75]. The precise set of events that govern which CpG residues are methylated is not understood, nor is the mechanism that causes DNA hypomethylation [76].

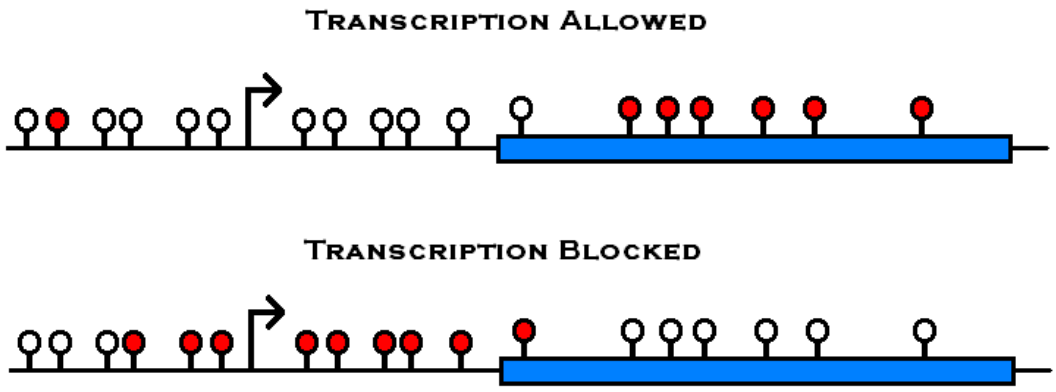

Figure 5 - Patterns of normal and altered promoter methylation. Normal transcription is allowed when little methylation is present surrounding the promoter, despite some upstream methylation and heavy methylation within the gene itself. Transcription becomes repressed when methylation surrounds the promoter. In cancerous cells this increase in methylation surrounding the promoter is accompanied by a decrease in methylation elsewhere in the gene.

In normal cells, repetitive elements such as long interspersed nucleotide elements (LINE), Alu repeats and satellite sequences make up almost half of the genome

and are methylated [77]. As these elements contribute largely to the level of global DNA methylation, it is no surprise that these regions are most drastically affected by hypomethylation, and as a result the stability that the DNA methylation once conferred to the chromosomes is lost. Supporting this is strong evidence that global hypomethylation plays a crucial role in causing genomic instability in colorectal carcinogenesis [78]. Such hypomethylation is observed in cancer cells and can be used as an indicator of genomic methylation levels [79]. Alternatively, gene specific DNA hypermethylation is another mechanism which can initiate carcinogenesis. This mechanism of gene silencing is demonstrated by the correlation of methylated gene promoters with a subsequent decrease in corresponding gene expression [80].

It appears that aberrant DNA methylation patterns are a cause rather than a consequence of the cancer as alterations can be identified in the early stages of cancer development [81]. For this reason, the mechanisms which regulate the methylation process have been highly sought after, yet remain elusive. There are several possible causes for the aberrant patterns of methylation observed in cancer and these are discussed below.

1.6.1 - DNMT expression

Among the most common explanations for the disturbance of regular methylation patterns in cancer cells is the up-regulation of the methyltransferase enzymes. It has been demonstrated that DNMT levels are elevated in several forms of cancer such as colorectal (*DNMT1* and *3B*) [82], endometrial (*DNMT3B*) [83], and general increase in methyltransferase activity has been

detected in leukaemia [84] and lung cancer [85]. Theoretically, the over-expression of methyltransferases could justify gene specific DNA methylation, but it does not account for the paradoxical genome wide hypomethylation. Even still, there is evidence to suggest that over expression of methyltransferases does not lead to promoter hypermethylation. Eads *et al.* demonstrated that the expression levels of DNMT1, 3A and 3B did not correlate with the frequency or extent of hypermethylation of *APC*, *ESR1*, *p16* or *MLH1* in colorectal adenocarcinomas [86]. Whilst methyltransferases were up-regulated when normalised with ß-actin or an RNA polymerase II large subunit, they were not significantly up-regulated when normalised with proliferation-dependent *H3F2* or *PCNA*. This suggests that although the methyltransferase levels appear increased in many cell types, they may in fact not be when they are normalised with other proliferation dependent genes.

Conversely, it has been argued that the down-regulation of maintenance methylation may lead to genome wide hypomethylation, however there is also evidence to refute this. Kimura *et al.* have shown that *DNMT1* methyltransferase gene expression was not correlated with the extent of DNA hypomethylation in transitional cell carcinomas; however, there was a decrease of *DNMT1* expression relative to cell proliferation [87]. In the same study, the authors addressed the hypermethylation of certain promoters, showing that DNMT3B levels were higher than in corresponding normal tissue, although DNMT3A levels were not.

Amid the evidence of altered levels and variations of expression levels, sometimes within the same cell type [88], it would not seem logical that simple

up or down regulation of one form of methyltransferase would cause site-specific hypermethylation in parallel with a global decrease in methylation. A co-ordinated breakdown involving both *de novo* and maintenance methyltransferases is more likely to explain the aberrant methylation patterns observed in cancer than methyltransferase expression levels.

1.6.2 - Subtle CpG Island Differences

Some CpG islands appear more frequently affected than others by methylation, supported by the observation that a cluster of genes displays hypermethylation in several forms cancer [89] that is absent in normal tissue. Not surprisingly the affected genes have critical roles in cells, including the maintenance of genomic integrity and tumour suppression. It would be reasonable to suggest that some CpG islands may be more likely to succumb to methylation based on CpG island size, GC content, CpG frequency, chromosomal location or promoter association. In work carried out in 2003 by Feltus *et al.*, the methylation status of several CpG islands was examined in cells over-expressing DNMT1 [90]. The majority of CpG islands tested were resistant to methylation, but a small proportion (3.8%) was found to be hypermethylated by DNMT1. Using this information, seven sequence patterns were identified that were capable of discriminating between prone and resistant CpG islands with a success rate of 87%. These sequences would appear to confer some form of susceptibility or resistance to methylation, possibly similar to the situation in which a non-methylated imprinted allele is resistant to methylation. In this study, the number of methylated CpG islands may have been biased, due to the over-expression of the maintenance methyltransferase rather than the *de novo* forms. Therefore the

hypermethylated regions in the study are in essence DNMT1 susceptible regions, and it is possible that the number of methylated islands would be higher if *de novo* methyltransferases were over-expressed. Another shortcoming of this study is that sequences alone will not define the methylation status of a particular gene, as non-methylated regions in normal tissue will also have the same sequence.

1.6.3 - Demethylation of DNA

The issue of active DNA de-methylation is currently an area of some controversy in the field of epigenetics. Should a bona-fide demethylase be recognised, it might provide explanations for many of the numerous epigenetic problems that arise in malignancy. Several groups have identified proteins that may be putative demethylases, however most of these have not been confirmed by data from other laboratories. As yet there does not appear to be a candidate that has profound demethylase activity without also having additional contradictory functions.

Several instances of possible demethylases have been reported [91-93] but there has been either little follow up or there is evidence to refute the original observations [94, 95]. The most controversial demethylase debate has surrounded the *MBD2* gene, with one group claiming it has de-methylating properties [96, 97] whilst others continue to find that it acts as a transcriptional repressor [98, 99]. Perhaps the strongest evidence presented regarding MBD2 demonstrates that mice lacking any functional copy of MBD2 are viable and show normal levels of genomic methylation [100], suggesting it is not necessary for demethylation, or at the very least, plays a small role in DNA demethylation.

More recently it has been reported that the DNA methyltransferases DNMT3A and DNMT3B are actively involved in the cyclical methylation of an active promoter, and that these enzymes exhibit deamination of methyl-cytosine [101, 102]. These two reports focus on the methylation and re-activation of the *pS2* gene in cultured breast cancer cells. It was shown that after induction, the promoter would undergo de-methylation and re-methylation within a 2 hour period which correlated with gene expression and recruitment of the methyltransferases to the promoters. Further studies are required to confirm these findings, in particular the kinetics with which the reaction is proposed to occur.

It might be possible that all the proposed mechanisms have some demethylating activity, but are only utilised by the cell under certain conditions. This might also explain why replication of such experiments by separate laboratories infrequently yields the same results.

1.6.4 - Dietary factors, including folate metabolism

There is a vast amount of evidence that nutrition obtained from dietary components has a major influence on individual health status, and there are at least two known pathways by which nutrient intake can affect DNA methylation. Firstly, the supply of nutrients affects the supply of methyl groups required for methylation. Numerous dietary components are known to influence DNA methylation status, and folate, choline, and vitamin B_{12} feature highly in the literature. Various forms of choline are converted into intermediates that are ultimately converted to S-adenosyl-methionine, the chemical substrate from which the methyltransferase enzymes obtain methyl groups for the attachment to

the DNA [103]. The precise role of folate and the effects of folate deficiency are complex, however it is thought to play an important function as a precursor methyl-donor. Vitamin B_{12} is a co-factor for many enzymatic processes leading to the methylation of DNA. Rats fed a diet deficient in Vitamin B_{12}, but not severe enough to cause illness, were observed to have hypomethylated genomic DNA in colon tissue compared to appropriate controls [104], illustrating that a diet deficient in Vitamin B12 can restrict DNA methylation.

The second mechanism by which diet can influence methylation relies on the effects of trace dietary components interfering with the methyltransferase process. A selenium deficiency has been shown to cause DNA hypomethylation in rat colon DNA, while prolonged cadmium exposure also initiates hypomethylation followed by hypermethylation, suggesting that a feedback loop is involved [105]. Nickel [106] and alcohol [107] have also been observed to affect DNMT activity in humans, although the precise mechanism of this is not understood.

1.6.5 - Methylation Spreading

The expansion of existing DNA methylation to cover neighbouring un-methylated sites is another hypothesis to explain aberrantly silenced genes. An experiment by Tollefsbol & Hutchinson has shown that using synthetic oligonucleotides, pre-existing methylation was able to spread to neighbouring CpG islands [108]. It was found that this pseudo-DNA with partial CpG methylation was more likely to undergo *de novo* methylation on non-affected CpG's than a control without pre-existing methylation. Interestingly, mammalian

methyltransferases were the only proteins necessary to induce this state, eliminating the notion that other factors are required for the expansion.

These results provide evidence which supports the spreading of methylation from ordinarily methylated DNA regions to areas which would not usually be methylated. The precise role that this plays in disease initiation is not known, and methylation spreading has weaker justification for the aberrant methylation observed in cancer, particularly global hypomethylation. However if spreading of methylation to normally unaffected regions occurs in conjunction with another mechanism, such as down-regulation of maintenance methylation, it could provide a clearer pathway in which aberrant disease-causing methylation patterns arise.

1.7 - Epimutations and the Two-hit Hypothesis

Knudson's two-hit hypothesis requires that both alleles of a tumour suppressing gene should be altered for disease progression to occur [109]. Germline mutations commonly represent the first hit of one allele, whilst the second hit typically arises from a sporadic mutation or loss of heterozygosity that affects the second allele. With the increasing detection of methylated promoters, refinements to Knudson's hypothesis can be made to accommodate epigenetic silencing. The *MLH1* gene is widely studied and will be used in the following examples. One such scenario of epigenetic silencing includes DNA methylation acting as the second hit, in unison with a pre-existing mutation on the second allele. This scenario has been detected on genes including *RB1*, *VHL*, *MLH1* and *BRCA1* [110-113]. A typical example of this is evident in the colorectal cancer cell line HCT116, which has a truncating mutation in one allele of the *p16* gene.

The wild-type allele is subjected to methylation whilst the mutated allele remains unmethylated, showing how these two mechanisms cooperatively silence genes [114]. A third mechanism involves both alleles, essentially the first and second 'hits', becoming deactivated by DNA methylation. Sporadic cases of colon cancer can sometimes be the result of bi-allelic methylation of *MLH1* [80]. Certain individuals have been shown to carry one methylated allele of a gene, which has been termed an 'epimutation' as it confers a similar risk of disease as if the allele were mutated [115].

The transmission of a methylated and repressed allele occurs mitotically allowing inheritance of stable patterns of gene expression, however it has recently been reported that a methylated allele can be transmitted from one generation to another. Chan *et al.* have shown *MSH2* epimutation can be inherited trans-generationally [116], however these subjects were mosaics. Interesting findings have been reported from Hitchins *et al.* who have identified two women with mono-allelic soma-wide DNA methylation of the *MLH1* gene [117]. The phenotype of these individuals was reported to mimic that of Hereditary Non Polyposis Colorectal Cancer (HNPCC), where germ line mutations typically occur in *MLH1* or *MSH2* genes (when a mutation is present in the mismatch repair system genes the condition is then referred to as Lynch Syndrome [118]). The first of these women has two sons, one of whom had inherited an identical haplotype to the mother, yet neither displayed the mono-allelic methylation. In the second woman identified with an *MLH1* epimutation, it was found that one of her four sons carried a methylated *MLH1* allele in somatic cells. The epimutation was not present in sperm cells from the affected

son, however, in a previous study it was reported that 1% of sperm from an affected male carried the epigenetic mark [119]. It has been suggested that epimutations might be more likely to arise or be maintained in oogenesis [117] and there is some plausible reasoning that supports this idea [120]. Hitchins *et al.* conclude that the transmission of the epimutation is likely the result of incomplete erasure or by retention of an epigenetic memory, but don't rule out *cis*-acting, or *trans*-acting factors which may render an individual more susceptible to epimutation.

1.8 - Epigenetic altering drugs

Given the control that epigenetic factors exert over the regulation of the genome, and coupled with the identification that these processes are altered in several disease states, components of the epigenetic pathway have become targets for several therapies against disease. A potent inhibitor of DNA methylation, 5-aza-2'-deoxycytidine, and a potent Histone Deacetylase Inhibitor, Trichostatin A are discussed here.

1.8.1 - 5-aza-2'-deoxycytidine (5-aza-dC)

5-aza-dC is an analogue of cytosine and is a strong inhibitor of DNA methylation (see figure 6). To exert its effect, the drug must first be incorporated into the DNA [121] during replication. 5-aza-dC is first transported into the cell by the facilitated nucleoside transport system [122] and then phosphorylated into a form which is then incorporated into DNA during replication [123]. Whilst attempting to methylate the 5-aza-dC substituted DNA, the methyltransferases form covalent adducts with the DNA and are firmly bound, with no detectable dissociation after 72 hours [124]. The amount of available methyltransferase

within the cell is then reduced, which in turn causes a reduction in genomic DNA methylation [125]. 5-aza-dC differs from cytosine at the carbon 5 position where a carbon is replaced by a nitrogen atom.

For some time it was unknown exactly why 5-aza-dC was toxic to cells and it was hypothesised that the inhibition of DNA methylation and subsequent induced hypomethylation may be responsible [126]. It was later proposed that re-expression of silenced genes involved with differentiation could result in terminal differentiation of leukaemic cells and may prove useful in therapy [127]. In 2005 it was reported that adducts created by the binding of *de novo* methyltransferases were stronger inducers of apoptosis than adducts formed by Dnmt1 binding [128], and is the most commonly accepted mechanism of 5-aza-dC toxicity.

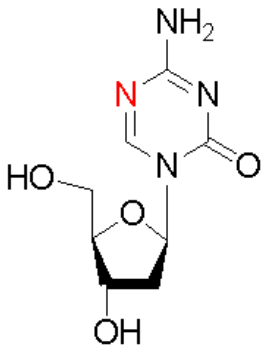

Figure 6 - The structural formula of 5-aza-dC. The carbon-5 position is highlighted showing the nitrogen atom in place of the carbon.

There are a number of tumour types where methyltransferase-inhibitor treatment may be beneficial, including bladder [129], breast [130], colon [131], lung [132], pancreas [133] and melanoma [134, 135]. The most promising outcomes have been displayed in the haematological malignancy myelodysplastic syndromes (MDS), whilst responses in solid tumour types have been below expectations

[136]. Effectiveness may be due to sensitisation to other reagents or re-activation of silenced genes which have an apoptotic effect. 5-aza-dC is marketed under the name Dacogen® for the treatment of MDS. In this setting, it is proposed that the drug can aid in normal bone marrow functioning and improve the symptoms of MDS [137].

1.8.2 - Trichostatin A (TSA)

Histone Deacetylase inhibitors have been touted as possible new anti-cancer therapies for over a decade [138, 139]. Trichostatin A is a potent inhibitor of Histone Deacetylase (HDAC) via binding to the catalytic domain [140] and was originally developed in 1976 as an antifungal agent [141] (see figure 7). In 1990 it was observed that treatment of cells with TSA lead to the accumulation of acetylated histones via inhibition of HDAC [142]. More recently, studies have shown that TSA affects the acetylation of histone H3 with little effect on histone H4, and is capable of inducing apoptosis through mechanisms which are now only beginning to be understood [143]. The apoptosis-inducing ability of TSA makes it useful as a cancer treatment, and although the mechanism of action remains unknown, it has been postulated that it acts via the reactivation of transcriptionally silent tumour suppressor genes, or modifying the expression of angiogenesis, apoptosis or cell cycles genes [144]. It has also been proposed that TSA may be useful in conjunction with other therapeutic agents such as Tamoxifen in oestrogen receptor α-negative breast cancer cells [145] or TRAIL in osteosarcoma cells [146]. Similarly there has been a synergistic effect related to treatment of cells with both 5-aza-dC and TSA with regard to particular gene expression, [60, 147, 148] but the effect is not as profound at a genome wide level [148].

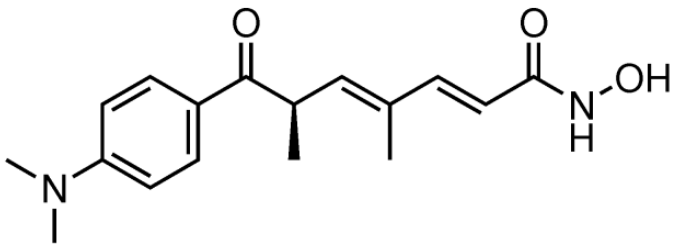

Figure 7 - The Structural formula of Trichostatin A.

1.9 - Rationale and Aims of this Study

Epigenetic control of gene expression is a complex process involving the co-ordination of several groups of proteins which function in a tightly regulated manner to ensure the appropriate genes are activated. The deregulation of correct epigenetic functioning displayed in tumor cells represents a major pathway in the initiation and progression of cancer. Despite an increasing knowledge of the proteins responsible for epigenetic modifications, there are numerous questions that remain unresolved.

The paradoxical DNA hypermethylation at promoter CpG islands whilst there is a concurrent genome wide hypomethylation at other regions remains difficult to explain. In addition the observation that this occurs in the majority of tumors suggests a common fault across all tumor types, and is yet to be elucidated.

The demethylating agent 5-aza-dC is an effective treatment of Myelodysplastic Syndrome. Whether the mode of action of this drug is due to the demethylating properties or cytotoxicity is unclear, as is its greater effectiveness on haematologic disorders in preference to other malignancies.

Long term exposure to 5-aza-dC is not a viable course of treatment, and the effect of the removal of the drug has not been adequately described. Importantly, the epigenetic components (including histone protein modifications) which are altered after 5-aza-dC and confer long term reactivation of a gene have not been identified.

Given that much remains unknown regarding the epigenetic control of gene expression, the aims of the studies described in this thesis include:

- Assess the role of a genetic variant which may influence DNA methylation and disease onset in individuals with Lynch Syndrome,
- To identify the role of DNA methylation patterns in expression of specific genes,
- To examine the effects of epigenetic altering drugs on genome wide and individual gene expression,
- To examine how DNA methylation levels and histone protein modifications respond during drug treatment to regulate gene expression
- Assess the gene pathways affected by 5-aza-dC exposure, and
- To identify which epigenetic modifications are important in maintaining the repressed or reactivated state of gene transcription.

It is anticipated that an increased understanding of the epigenetic regulation of gene expression will contribute in part to further comprehension of the molecular basis of diseases such as cancer, and identify novel targets for new treatments.

Chapter 2

The -149C>T SNP within the *ΔDNMT3B* gene is not associated with early disease onset in Hereditary Non-Polyposis Colorectal Cancer

STATEMENT I

This statement explains the contribution of all authors in the article listed below:

Reeves, S.G., Mossman, D., Meldrum, C.J., Kurzawski, G., Lubinski, J., and Scott, R.J., (2008) The -149C>T SNP within the *ΔDNMT3B* gene is not associated with early disease onset in hereditary non-polyposis Colorectal Cancer. *Cancer Letters*, 265(1):39-44.

Table I: Author contribution Percentage and Description of Contribution to the article listed above.

Author	**Contribution (%)**	**Description of Contribution to Article**	**Signature**
Stuart G. Reeves	45%	Experimental design, executed the experiment, co-performed statistical analysis. Co-wrote the manuscript.	
David Mossman	40%	Experimental design, executed the experiment, co-performed statistical analysis. Co-wrote the manuscript.	
Cliff J. Meldrum	2.5%	Provided samples and clinical information.	
Grzegorz Kurzawski	2.5%	Provided samples and clinical information.	
Janina Suchy	2.5%	Provided samples and clinical information.	
Jan Lubinski	2.5%	Provided samples and clinical information.	
Rodney J. Scott	5%	Designed the study, provided the concept and corrected the manuscript.	

Chapter Introduction:

During 2006, a report by Jones *et al.* was published claiming that a single nucleotide polymorphism within the *DNMT3B* gene influenced the age of onset of colorectal cancer in a group of individuals with a predisposition to Hereditary Non-Polyposis Colorectal Cancer. This was of particular interest as it specifically implies that an alteration to the DNA methyltransferase Δ*DNMT3B* gene is altering disease expression, although a specific epigenetic alteration was not identified in the original paper by Jones *et al*. The Δ*DNMT3B* SNP may represent a mechanism by which DNA methylation may act as a modifier of disease development. We sought to investigate the findings of Jones *et al.* as it may contribute to the understanding of epigenetic regulation of gene expression and is therefore of relevance to the overall aims of this thesis.

Our experimental findings did not support previous studies that a Δ*DNMT3B* SNP could alter the age of onset on HNPCC. We concluded that a Type I error was responsible for reported effect observed by Jones *el al.* that was not observed in our experiment due to the larger sample size.

This study was performed jointly by myself and Stuart Reeves. My role in this study was to identify the exact genomic loci in question, as none of the previous reports concerning this SNP used the same gene name variant, despite reference to the previous studies. In the laboratory component, I performed an equal amount of the PCRs, enzymatic digestions, gel electrophoresis and data interpretation. However, Stuart performed a greater proportion of the statistical analysis and as a result is entitled to a greater share of the authorship of this manuscript.

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The −149C>T SNP within the ΔDNMT3B gene, is not associated with early disease onset in hereditary non-polyposis colorectal cancer

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Abstract

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited syndrome caused by germline mutations in mismatch repair (MMR) genes. HNPCC patients have a lifetime risk of 80% of developing colorectal cancer (CRC); however the likely age of onset is difficult to predict. A single C>T polymorphism located within the promoter region of the ΔDNMT3B gene has recently been reported to be associated with a significant increase to the risk of early onset CRC. In this study we determined the ΔDNMT3B genotype in 404 confirmed HNPCC participants (total of 194 CRC cases) from Australia (203) and Poland (201). From the total number of participants there were 194 diagnosed cases of CRC and 210 healthy MMR gene mutation carriers. The study was undertaken to assess whether the reported effect observed in a previous study of 146 HNPCC patients is consistent in a larger separate and unrelated participant cohort. Through the statistical tests of Kaplan–Meier survival analysis and Cox hazard regression models we did not observe any significant association between the ΔDNMT3B C>T SNP and early onset CRC in HNPCC patients.

Keywords: DNMT3B; Hereditary non-polyposis colorectal cancer; Disease expression; Epidemiology

1. Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) is the most common form of hereditary colorectal cancer (CRC), accounting for approximately 2–5% of all CRC cases [1]. The disease is due to either a loss or reduced function of one of the DNA mismatch repair (MMR) genes MLH1, MSH2, MSH6 or PMS2 [2]. A reduction in the fidelity of MMR results in an increased likelihood of colorectal tumours and a heightened risk of other epithelial malignancies [3]. The MMR genes MLH1 and MSH2 are the most frequently mutated;

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however, insult to these genes is not limited to HNPCC. Approximately 15–20% of sporadic cancers display features that are suggestive of MLH1 silencing [4] likely to have arisen from aberrant DNA methylation.

Three methyltransferase enzymes, namely DNMT1, 3A and 3B catalyse the DNA methylation process in humans. The DNMT1 form is largely responsible for duplicating the methylation pattern on the newly synthesized DNA strand following replication. The *de novo* methyltransferases, DNMT3A and 3B, however are thought to establish and co-ordinate methylation during times of development and in imprinting [5]. Due to their mechanism of action, it is thought that the *de novo* methyltransferases are responsible for the aberrant methylation of promoters. Expression of DNMT3A and 3B is elevated in multiple forms of cancer [6–8], and the DNMT3B methyltransferase levels have been shown to be 3.7-fold greater in colon tumour tissue compared to normal surrounding tissue [9].

Four enzymatically active variants of the DNMT3B gene have been identified namely, DNMT3B1, 3B2, 3B3 and 3B6. In 2006, a new isoform, ΔDNMT3B, has been identified and has been shown to be the predominant form of DNMT3B expressed in non-small cell lung cancer cell lines [10]. It is stated that the ΔDNMT3B version uses an alternate promoter located within intron 4 and exon 5 of the DNMT3B gene.

In 2004 it incorrectly reported that a C>T single nucleotide polymorphism (SNP) was present within the promoter of DNMT3B6 [11], as the SNP does not fall within the promoter region of DNMT3B6 (Accession No. NM_175850.1). In 2006 the same SNP was again incorrectly reported to fall within the promoter of DNMT3B [12] but this does not match the Genbank record for DNMT3B (Accession No. NM_006892.3). Both of these reports are referring to the same SNP that is within the promoter of the ΔDNMT3B isoform (located at position 1570351, Accession No. NT_028392), −149 bp from the transcription start site of ΔDNMT3B, which falls on intron 4 of the regular DNMT3B gene. The T variant of this SNP has been associated with a 2-fold increase in promoter activity [13], and the ΔDNMT3B C>T SNP has been reported to play a role in the age of onset of colorectal cancer [12], where it has been proposed that the T variant allele results in increased ΔDNMT3B expression, and aberrant *de novo* methylation, which is expressed as an earlier age of cancer onset.

In this study we have further investigated the role of the ΔDNMT3B SNP and its association with the age of colorectal cancer onset in 404 MSH2 and MLH1 mutation positive participants. Using restriction fragment length polymorphism analysis, Kaplan–Meier statistics and Cox hazard regression models we re-assessed the correlation between the ΔDNMT3B C>T SNP and early onset CRC in HNPCC patients.

2. Methods

2.1. Patients

All participants included in this study were enrolled after they had been evaluated at a family cancer clinic and were diagnosed with HNPCC. The selection criteria used for enrolment into this study was strictly defined on the basis of a molecular diagnosis of this syndrome due to the presence of a causative germline mutation in a DNA mismatch repair gene. Our study included a total of 404 participants (194 CRC cases) including 203 Australian and 201 Polish cases of which there were 143 unrelated probands and 261 patients from 69 families all with confirmed hMLH1 or hMSH2 germline mutations. The Institutional Ethics review boards of the Pomeranian Academy of Medicine and the Hunter New England Health Service approved the study. All participants gave written informed consent for the DNA samples to be used for research into HNPCC.

The clinical and demographic characteristics of the participants used in this study are shown in Table 1.

2.2. PCR conditions and C>T SNP analysis

The region containing the C>T SNP located −149 bp from the transcription start site of ΔDNMT3B were genotyped using the same primers as described previously by Jones et al. [12]. A 376 bp fragment spanning the region surrounding the SNP was generated using Polymerase Chain Reaction (PCR). Reactions were performed using 50 ng of genomic DNA, 1× PCR buffer, 0.2 mM dNTP, 1 mM $MgCl_2$, 1.2 μM of each primer and 0.2 U of Platinum Taq Polymerase (Invitrogen). Thermal cycling conditions involved a denaturation step at 94 °C for 5 min, 14 cycles of 94 °C for 30 s, 63 °C for 45 s (decreasing by 0.5 °C per cycle to 56.5 °C), 72 °C for 1 min then 20 cycles at the above conditions with an annealing temperature of 56 °C. This was followed by a final extension step at 72 °C for 10 min.

PCR products were digested for 16 h at 37 °C with the restriction enzyme AvrII (New England Biolabs) before being run on a DNA electrophoresis gel to determine results. Digestions were performed using 3 μl PCR product, 1× buffer (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothritol), and 0.6 U of AvrII. This

Table 1
Clinical and demographic characteristics of study participants

Characteristic	Colorectal cancer	No colorectal cancer	Total $n = 404$
Population			
Australian	98	105	203
Polish	96	105	201
Gender			
Female	113	139	252
Male	81	71	152
Age			
Mean	–	46.5	46.5
Range	–	18–95	18–95
Age of CRC			
Mean	42.7	–	42.7
Range	16–78	–	16–78
Proband			
Yes	87	56	143
No	109	152	261
MMR mutation			
MLH1	108	128	236
MSH2	86	82	168
Mutation type			
Truncation/ deletion	171	173	344
Missense	23	37	60

*All missense mutations included were deemed to be pathogenic through previous functional assay studies. Age for participants without colorectal cancer is defined as age at last follow up.

cut the 376 bp PCR product only when the C>T SNP was present, producing two bands (136 bp and 240 bp) for a homozygote C>T change or three bands (376 bp, 240 bp and 136 bp) if a heterozygote change was present. For wild-type C/C genotypes the 376 bp product remained intact. Genotyping was repeated in a total of 5% of participants to confirm the original analysis; re-genotyping revealed a concordance rate of 100%.

2.3. Statistical analysis

All statistical analyses were completed using Intercool Stata 8.2 (Stata Corp, College Station, TX).

Kaplan–Meier (KM) analysis was initially used to conduct univariate analysis with models including MMR mutation type, and gender compared to the age of clinical onset of CRC in relation to the ΔDNMT3b C>T SNP genotype. Through several statistical tests including the long rank test, KM was able to identify the proportion of the patient population that are cancer free at the age of evaluation compared to those patients who have developed disease.

Cox proportional hazard regression models were employed to determine the association of early onset colorectal cancer risk with multiple variables. These models allowed us to take into consideration the variables of: gender, MLH1/MSH2 mutation group, ethnicity and ΔDNMT3b genotype in regards to family clustering (i.e. groups of participants belonging to the same family). *p*-Values generated through Cox modelling were supported with hazard ratios (HR) and 95% confidence intervals (CI) giving an overall more robust model.

The age of diagnosis was defined as the patient's age at the time of colorectal cancer diagnosis, whereas the age for unaffected MMR gene carriers was determined by using their date of birth and disease free status at last follow-up, which was treated as censored in the analysis.

3. Results

Utilising the technique of PCR a 376 bp fragment was generated. Restriction enzyme digestion followed by agarose gel electrophoresis allowed three different ΔDNMT3B genotypes (CC, CT, TT) to be identified as shown in Fig. 1.

Participants were genotyped for ΔDNMT3B by this method before results were tabulated. Table 2 shows a summary of the patient demographics included in the study, including CRC status, gender, mismatch repair mutation and type, ethnicity and allele frequency by ΔDMNT3B genotype. Both populations were assessed separately and found to be in Hardy–Weinberg Equilibrium (HWE). When analysed as a single population, the allele frequencies remained in HWE ($p = 0.18$).

KM analysis was performed on all genotypes generated to determine whether any significant differences between the ΔDNMT3B C>T SNP and early onset disease were present. P values were determined using the Log Rank (LR) test, which gives equal weight to all failures, Wilcoxons (W) test, which emphasises observations from early onset patients, and finally the Taron–Ware (TW) test that gives an intermediate of the LR and W tests. Table. 3.

The three different ΔDNMT3B genotypes (CC, CT, TT) were first individually tested for all participants however no significant result was found overall (LR, $p = .83$), (W, $p = .82$) and (TW, $p = .77$). We then divided the patient data into two more general groups namely wild-type homozygous (CC) and combined heterozygous/homozygous (CT or TT) SNP. This again however

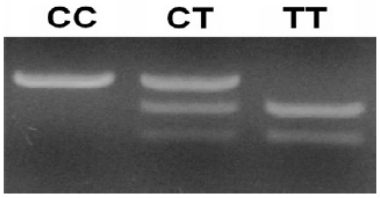

Fig. 1. DNMT3b genotypes (CC, CT, TT) generated by AvrII restriction enzyme digestion.

Table 2
Patient demographics by DNMT3B genotype

	CC, *n* (%)	CT, *n* (%)	TT, *n* (%)	Total
Colorectal cancer				
Yes	57(29.4)	91(47.0)	46(23.6)	194
No	63(30.0)	97(46.2)	50(23.8)	210
Gender				
Male	48(31.6)	70(46.0)	34(22.4)	152
Female	72(28.6)	119(47.2)	61(24.2)	252
MMR gene				
MLH1	68(28.8)	111(47.0)	57(24.2)	236
MSH2	52(31.0)	77(45.8)	39(23.2)	168
MMR mutation type				
Truncation/ deletion	106(30.8)	157(45.6)	81(23.5)	344
Missense	14(23.3)	31(51.7)	15(25.0)	60
Ethnicity				
Australian	61(30.0)	93(45.8)	49(24.2)	203
Polish	59(29.3)	95(47.3)	47(23.4)	201
Allele frequency				
C allele				0.5347
T allele				0.4653

Table 3
Hazard ratios and 95% confidence intervals by ΔDNMT3B genotype

Genotype	HR	95% CI	*p*-value
CT	0.94	[0.66, 1.31]	0.70
TT	0.94	[0.63, 1.42]	0.78
CT + TT	0.94	0.68, 1.29]	0.70
Per allele dose model	0.97	[0.79, 1.12]	0.77

Reference group for CT, TT and CT + TT is CC.
Per allele dose model refers to the individual CC, CT and TT genotypes.

showed no significant *p*-values for early onset disease associations (LR, $p = .54$), (W, $p = .63$), (TW, $p = .51$). Kaplan–Meier plots of these results are shown below in Fig. 2.

No significant difference was also observed in the separate population groups (Australian vs. Polish) or in the specific MMR mutation groups (MLH1 vs. MSH2), data not shown.

Cox regression models were then used to verify results shown by KM as well as to take into account any variables that may prove to be significant. Cox analysis on the individual genotypes (CC, CT, TT) along with the heterozygote, homozygote and combined heterozygote/homozygote forms were tested using the wild-type allele as a reference. All models included in this analysis contained gender and family clustering as additional variables resulting in non-significant *p*-values with hazard ratios pointing in the opposite direction as opposed to Jones

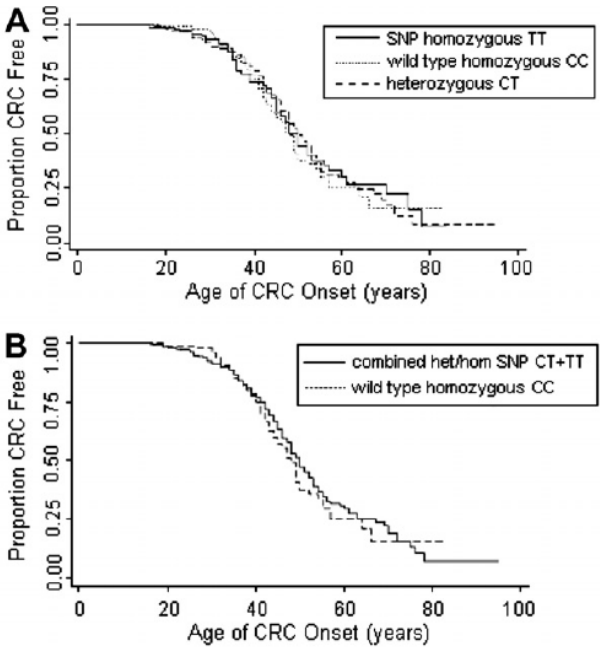

Fig. 2. Kaplan–Meier survival analysis by ΔDNMT3B genotypes The plots show the non-significant effect of the ΔDNMT3B genotype on age of onset for CRC in HNPCC patients. (A) By specific genotype and (B) by CT and TT genotypes compared to CC wild-type.

et al. [12]. Additionally, dividing participants into groups of ⩽40 vs. >40 did not alter this non-significant association for either individual (CC, CT, TT) or combined (CC vs. CT/TT) genotypes. Individual gender affects along with separate populations and MMR mutation groups were also included in additional Cox models. These also confirmed the initial KM results showing no significant association overall.

4. Discussion

We have analysed the ΔDNMT3B C>T SNP in 404 HNPCC participants to determine if the genotype correlates with the age of onset of colorectal cancer. Our results suggest no association between this SNP and age of CRC onset in our study populations.

This is inconsistent with the results published by Jones et al. [12] where a significant difference in age of onset between wild-type, heterozygous and homozygous mutant carriers in the ΔDNMT3B gene was reported. We utilised KM survival analysis and Cox hazard regression models to assess for differences amongst the three genotypes and additionally to compare wild-type against the combined heterozygotes and homozygote forms. Our results show the ΔDNMT3B C>T SNP did not alter age

of onset in either the Australian or Polish population, and no effect was observed when patients were grouped based on the type of MMR mutation they harboured (i.e. MLH1 or MSH2). The LR, W and TW tests were all used to assess for significant differences at different time points of the KM survival curves. The results of these tests however suggested no significant association for earlier disease onset age.

Cox regression modelling that included gender, MMR group and family clustering as variables also confirmed the KM results for no significant association for each individual genotype or for the homozygous/heterozygous C>T SNP versus wild-type. This was found to be the case for individual population groups (Australia and Poland) and additionally when both groups were combined together.

The ΔDNMT3B promoter SNP lies within an intronic region between the fourth and fifth transcribed exons of the longest isoform of the DNMT3B gene (DNMT3B1). It has been demonstrated that this SNP causes an increased level of expression of the ΔDNMT3B gene [13], and whilst it has been proposed that this SNP causes an earlier age of disease onset in HNPCC patients, our results provide evidence to suggest this is not the case. It is plausible however, that basic expression of ΔDNMT3B, rather than the presence of the ΔDNMT3B C>T SNP, is related to earlier age of disease onset. Wang et al. [10] reported that ΔDNMT3B is the predominant form of DNMT3B expressed in non-small cell lung carcinoma, and that a smaller percentage of matched healthy tissues express the ΔDNMT3B form of the gene. In this case, a patient who carries the ΔDNMT3B T allele may not develop disease any earlier than a patient with the C allele, as the ΔDNMT3B form is not expressed. This may also explain the discrepancies between our results and that of Jones et al. [12].

Considering the 3-fold larger participant size in this study, it is likely that a type 1 error may account for the discrepancy of results between these two studies. Jones et al. included only 12 confirmed CRC patients who carried the CC wild-type allele, 45 with a heterozygote CT and 17 patients with the homozygote TT allele. Statistical tests on such few patients in each category are therefore more likely to give an erroneous result due to the small sample size. Our cohort however contained 57 CC, 91 CT and 46 TT genotypes in patients with CRC; therefore the larger sample size supports greater strength to our statistical analysis.

Potential limitations of the current study may include population stratification however; we believe this should not affect overall outcomes as a true modifying polymorphism will affect disease expression in all HNPCC patients independent of population group. Additionally, no significant effect was observed for this SNP in either the Australian or Polish population by Cox analysis (results not shown) providing support of limited population stratification confounding effects. Different environmental influences on CRC onset age between the two countries, could potentially affect the results, but again this is unlikely as the average age of onset for the Australian and Polish groups were 42.8 and 43.5 years, respectively. The potential for individual environmental effects however cannot be entirely ruled out. Another limiting factor in this study is the reduced power to detect any small moderate effects that may be occurring. Despite being one of the larger HNPCC participant cohort studies, it would be beneficial to examine larger HNPCC populations thereby providing greater statistical rigour.

In conclusion, we have found no significant association for earlier cancer onset age in the Australian and Polish HNPCC populations or in a combined population cohort of 404 participants. Our findings lead us to conclude that there is no distinct correlation between the C>T SNP in the ΔDNMT3B promoter and early onset CRC in HNPCC patients and believe previous results reporting a positive association may be due to a type 1 error.

Acknowledgments

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References

[1] H.T. Lynch, T.C. Smyrk, P. Watson, S.J. Lanspa, J.F. Lynch, P.M. Lynch, R.J. Cavalieri, C.R. Boland, Genetics, natural history, tumour spectrum, and pathology of hereditary nonpolyposis colorectal cancer: and updated review, Gastroenterology 104 (5) (1993) 1535–1549.

[2] H.T. Lynch, A. de la Chapelle, Genetic susceptibility to nonpolyposis colorectal cancer, Journal of Medical Genetics 36 (1999) 801–818.

[3] M. Aarnio, R. Sankila, E. Pukkala, R. Salovaara, L.A. Aaltonen, A. de la Chapelle, P. Peltomaki, J.P. Mecklin, H.J. Jarvinen, Cancer risk in mutation carriers of DNA-mismatch repair genes, International Journal of Cancer 81 (2) (1999) 214–218.

[4] S.N. Thibodeau, G. Bren, D. Schaid, Microsatellite instability in cancer of the proximal colon, Science 260 (1993) 816–819.
[5] H.D. Morgan, F. Santos, K. Green, W. Dean, W. Reik, Epigenetic reprogramming in mammals, Human Molecular Genetics 1 (14 review issue) (2005) r47–r58.
[6] J.R. Melki, P. Warnecke, P.C. Vincent, S.J. Clark, Increased DNA methyltransferase expression in leukemia, Leukemia 12 (3) (1998) 311–316.
[7] F. Jin, S.C. Dowdy, Y. Xiong, N.L. Eberhardt, K.C. Podratz, S.W. Jiang, Up-regulation of DNA methyltransferase 3B expression in endometrial cancers, Gynaecological Oncology 96 (2) (2005) 531–538.
[8] S.A. Belinsky, K.J. Nikula, S.B. Baylin, J.P. Issa, Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer, Proceedings of the National Academy of Science, USA 93 (9) (1996) 4045–4050.
[9] C. Schmutte, A.S. Yang, T.T. Nguyen, R.W. Beart, P.A. Jones, Mechanisms for the involvement of the DNA methylation in colon carcinogenesis, Cancer Research 56 (1996) 2375–2381.
[10] L. Wang, J. Wang, S. Sun, M. Rodriguez, P. Yue, S.J. Jang, L. Mao, A Novel DNMT3B subfamily, ΔDNMT3B, is the predominant form of DNMT3B in non-small cell lung cancer, International Journal of Oncology 29 (2006) 201–207.
[11] L. Wang, M. Rodriguez, E.S. Kim, Y. Xu, N. Bekele, A.K. El-Naggar, W.K. Hong, L. Mao, Y.W. Oh, A Novel C/T polymorphism in the core promoter of human de novo cytosine DNA methyltransferase 3B6 is associated with prognosis in head and neck cancer, International Journal of Oncology 25 (4) (2004) 993–999.
[12] J.S. Jones, C.I. Amos, M. Pande, Z. Gu, J. Chen, I.M. Campos, Q. Wei, M. Rodriguez-Bigas, P.M. Lynch, M.L. Frazier, DNMT3b polymorphism and hereditary nonpolyposis colorectal cancer age of onset, Cancer Epidemiology Biomarkers and Prevention 15 (5) (2006) 886–889.
[13] H. Shen, L. Wang, M.R. Spitz, W.K. Hong, L. Mao, Q. Wei, A novel polymorphism in human cytosine DNA-methyltransferase-3B promoter is associated with an increased risk of lung cancer, Cancer Research 62 (2002) 4992–4995.

Chapter 3

Demethylation by 5-aza-2-deoxycytidine in Colorectal Cancer Cells Targets Genomic DNA whilst promoter CpG island methylation persists

STATEMENT II

This statement explains the contribution of all authors in the article listed below:

Mossman, D., Kim, K.T., and Scott, R.J., (2009) Demethylation by 5-aza-2-deoxycytidine in Colorectal Cancer Cells Targets Genomic DNA whilst promoter CpG island methylation persists. BMC Cancer 2010, **10:**366.

Table II: Author contribution Percentage and Description of Contribution to the article listed above.

Author	**Contribution (%)**	**Description of Contribution to Article**	**Signature**
David Mossman	85%	Executed the study. Analysed and interpreted the data. Wrote the manuscript.	
Kyu-Tae Kim	5%	Assisted with the apoptosis and cytotoxicity assay.	
Rodney J. Scott	10%	Designed the study, provided the concept and corrected the manuscript.	

Chapter Introduction:

The *ΔDNMT3B* SNP studied in Chapter 1 did not appear to modify the age of disease onset in HNPCC mutation carriers, so alternative methods to investigate the process of DNA methylation were developed with a cell line model. In the experiments described in Chapter 2, we sought to investigate how the levels of genomic and gene specific DNA methylation would return after treatment with the methyltransferase inhibitor 5-aza-dC in colorectal cancer cell lines. We were also able to assess genome wide changes in gene expression and correlate this with the methylation of specific genes, and identify methylation patterns which affect gene expression. The treatment of solid tumours with methyltransferase inhibitors is less effective than anticipated, and this work identifies a DNA methylation pattern that may govern whether a particular gene is reactivated transiently or more long term after drug treatment. This may represent a type of biomarker which can predict whether a methylated and suppressed gene can be successfully reactivated long term with drug treatment. The conclusions of this study highlighted that demethylation induced by 5-aza-dC exposure caused little change in the DNA methylation of specific genes despite a large increase in transcription and that the pattern of pre-existing DNA methylation may play an important role in gene reactivation.

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RESEARCH ARTICLE Open Access

Demethylation by 5-aza-2'-deoxycytidine in colorectal cancer cells targets genomic DNA whilst promoter CpG island methylation persists

David Mossman[1,2], Kyu-Tae Kim[1,2] and Rodney J Scott*[1,2,3]

Abstract

Background: DNA methylation and histone acetylation are epigenetic modifications that act as regulators of gene expression. Aberrant epigenetic gene silencing in tumours is a frequent event, yet the factors which dictate which genes are targeted for inactivation are unknown. DNA methylation and histone acetylation can be modified with the chemical agents 5-aza-2'-deoxycytidine (5-aza-dC) and Trichostatin A (TSA) respectively. The aim of this study was to analyse de-methylation and re-methylation and its affect on gene expression in colorectal cancer cell lines treated with 5-aza-dC alone and in combination with TSA. We also sought to identify methylation patterns associated with long term reactivation of previously silenced genes.

Method: Colorectal cancer cell lines were treated with 5-aza-dC, with and without TSA, to analyse global methylation decreases by High Performance Liquid Chromatography (HPLC). Re-methylation was observed with removal of drug treatments. Expression arrays identified silenced genes with differing patterns of expression after treatment, such as short term reactivation or long term reactivation. Sodium bisulfite sequencing was performed on the CpG island associated with these genes and expression was verified with real time PCR.

Results: Treatment with 5-aza-dC was found to affect genomic methylation and to a lesser extent gene specific methylation. Reactivated genes which remained expressed 10 days post 5-aza-dC treatment featured hypomethylated CpG sites adjacent to the transcription start site (TSS). In contrast, genes with uniformly hypermethylated CpG islands were only temporarily reactivated.

Conclusion: These results imply that 5-aza-dC induces strong de-methylation of the genome and initiates reactivation of transcriptionally inactive genes, but this does not require gene associated CpG island de-methylation to occur. In addition, for three of our selected genes, hypomethylation at the TSS of an epigenetically silenced gene is associated with the long term reversion of gene expression level brought about by alterations in the epigenetic status following 5-aza-dC treatment.

Background

DNA methylation is an epigenetic modification that occurs on cytosine residues in the sequence context 5'-CG-3'. It is well established that DNA methylation acts as a transcriptional repressor of gene expression via recruitment of repressive proteins. These include the Methyl-CpG Binding Protein 1 (MeCP1) and proteins with a methyl-binding domain, such as MBD1, MBD2, MBD3, MBD4 and MeCP2. These proteins hinder transcription through the recruitment of other factors such as nucleosome remodelling complex [1]. In the case of MeCP2, the protein is capable of binding to a single symmetrically methylated cytosine and contributing to the long-term repression of transcription [2]. The binding of these additional protein factors leads to condensation of DNA and confers stability to the chromosome.

In normal cells, repetitive elements such as long interspersed nucleotide elements, Alu repeats, transposable elements, and satellite and non-satellite sequences which together make up almost half of the genome, are methylated [3-5]. Methylation of these regions largely contrib-

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utes to the level of global methylation, and it is likely that these regions are most drastically affected by aberrant hypomethylation and the stability that the methylation once conferred to the chromosomes is lost. Aberrant methylation is one of the more frequent molecular changes observed in tumour cells [6] and typically involves the reversal of normal methylation patterns. It has been known for some time that common changes involve genome wide hypomethylation, which impinges on the expression of oncogenes [7], loss of imprinting and hypermethylation of tumour suppressor genes [8]. These are believed to be a cause rather than a consequence of the malignant process as they arise early in disease development [9]. Supporting this is strong evidence that global hypomethylation plays a crucial role in causing genomic instability in colorectal carcinogenesis [10]. Alternatively, gene specific hypermethylation is another mechanism which can initiate carcinogenesis. This mechanism of gene silencing has been shown by the correlation of methylated promoters with a subsequent decrease in corresponding gene expression. The precise set of events that govern which CpG residues are methylated are not understood, nor is the mechanism that causes hypomethylation [11].

5-aza-2'-deoxycytidine (5-aza-dC) is a strong inducer of DNA de-methylation. It is an analogue of cytosine, that when incorporated into DNA, irreversibly binds the methyltransferase enzymes as they attempt to methylate the cytosine analogue. This depletion of methyltransferase in the cell results in passive de-methylation, which is known to reactivate epigenetically silenced genes [12]. 5-aza-dC has demonstrated its most positive effect in the treatment of hematologic malignancy such as myelodysplastic syndromes [13]. In this scenario, its effectiveness may be due to sensitisation to other reagents or the reactivation of silenced genes which have an apoptotic effect. Another agent which affects the epigenetic status of genes is Trichostatin A (TSA). TSA was originally developed as an antifungal agent [14], but was also found to lead to the accumulation of acetylated histones via the inhibition of Histone Deacetylase [15]. The presence of an acetyl group on a lysine amino acid in the N-terminal end of core histone proteins neutralises the positive charge carried by the lysine, weakening the association between the nucleosome and DNA [16] to favour transcriptional activity. Reports have demonstrated a synergistic effect of TSA with 5-aza-dC in the re-expression of epigenetically silenced genes [17-20]. Currently it is not known how methylation patterns are altered with 5-aza-dC, and how or if these patterns can be restored when drug treatment ceases.

The aim of this study was to examine patterns of DNA methylation in several colorectal cancer (CRC) cell lines, to assess how these patterns are affected by drugs which alter epigenetic status, and profile the re-methylation process at both a genome-wide and gene specific level. Currently, the process of re-methylation following 5-aza-dC is not well documented.

Methods

Cell Culture

Colorectal cancer cells HCT116, SW48, SW480, HT29 and a fibroblast cell line derived from a healthy donor were all cultured in DMEM supplemented with 10% Foetal Calf Serum (Sigma-Aldrich, St Louis, MO) at 37°C and 5% CO_2. De-methylation was induced with 5-aza-dC (Sigma-Aldrich) treatment at a pre-determined concentration that induced maximal de-methylation of the DNA without killing the cells. Culture media for LoVo and the fibroblast cells contained 10 μM 5-aza-dC, whilst all other cells were treated with 15 μM. Cells were incubated for 72 h with 5-aza-dC with the culture media being replaced every 24 h with fresh media containing 5-aza-dC. DNA and RNA were extracted before drug treatment and after 72 h of drug treatment. Immediately following drug treatment (72 h), a fraction of the cells were washed twice with PBS and allowed to continue growing under regular drug-free conditions. At every two days following cessation of treatment, DNA and RNA were extracted while a fraction of the cells continued to be incubated until ten days of drug free growth. The experiment was also performed on HCT116 cells continuously exposed to 150 ηM Trichostatin A (Sigma-Aldrich) during the treatment and re-methylation period to assess the affect of histone acetylation on DNA re-methylation. This concentration chosen has previously been shown to cause hyperacetylation in the HCT116 cell line [21].

Cytotoxicity and Apoptosis testing

The assessment of cytotoxicity and apoptosis was undertaken using pooled cell cultures as per the assay protocol provided with the detection kits. For practicality, the HCT116, SW480 and LoVo cell lines were assayed. Cytotoxicty was quantified using Cytotoxicty Detection Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Apoptosis was determined using an Annexin V Apoptosis Detection Kit from BD Biosciences and analysed on a BD FACSCantoII flow cytometer (Becton Dickinson, Franklin Lakes, NJ) following the manufacturer's instructions.

High Performance Liquid Chromatography (HPLC) Analysis of Global Methylation Levels

50 μg of DNA was treated with 5 μL RNAse Cocktail (Ambion, Austin, TX) to remove any residual RNA which would interfere with HPLC analysis. DNA was then phenol-chloroform extracted and resuspended in sterile water. DNA aliquots of 3 μg were digested with 1.5 U of

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Nuclease P1 (US Biological, Swampscott, MA) and incubated at 37°C for 16 h. Following digestion, 2 μL of Calf Intestinal Alkaline Phosphatase (Promega, Madison, WI) was added and incubated at 37°C for a further two hours. Separation of nucleosides was performed on a Varian Star Chromatography workstation with a Supelcosil LC-18-DB column (Sigma-Aldrich) over 30 min at 35°C with absorbance monitored at 278 nm. Peak areas were quantified with Star Reviewer Software (Varian, Palo Alto, CA) and the 5-methylcytosine content was expressed as a percentage of the total cytosine pool after correction for extinction co-efficients. Standard deviations were calculated and a T-test was employed to compare expression levels in drug treated cells against untreated cells. *P*-values less than 0.05 were considered to be statistically significant.

Illumina arrays and Analysis

Illumina Human Ref-8 expression arrays (Illumina, Hayward, CA) were used to measure genome wide gene expression at four chosen time points; untreated cells, treated cells (day 0 of re-methylation), and at four (d4) and ten days (d10) of drug free growth and re-methylation. These time points were selected based on global methylation levels at these time points. Untreated and treated cells were used to identify changes caused by 5-aza-dC, whilst day 4 would allow an intermediate and day 10 a final assessment of the changes in gene expression during re-methylation. Data analysis was performed in Genespring 7.3.1. software (Agilent, Foster City, CA). Genes were classified according to their expression pattern, and were selected with the aim of comparing methylation patterns in genes temporarily reactivated and those still expressed after 10 days of drug free growth. The genes selected for analysis were *CDO1, HSPC105, MAGEA3, RNF113B., ZFP3* and two colorectal cancer related genes *CDKN2A* and *MLH1*. These genes displayed different expression patterns in the HT29, SW480, SW48 and HCT116 cell lines that could be further examined.

Bisulfite conversion, PCR and direct sequencing

DNA was converted in duplicate using a Qiagen Epitect Bisulfite conversion kit (Qiagen, Valencia, CA) using 2 μg of phenol-chloroform purified DNA. Samples were eluted in 30 μL of elution buffer and an aliquot was diluted 1:3 prior to PCR and stored at 4°C, whilst the remaining fraction was stored at -20°C. CpG islands surrounding the transcription start site of genes were targeted in PCR analysis using the primers listed in Additional File 1: Table S1. Primers included a non-CpG cytosine at the 3' end in order to preferentially amplify converted DNA sequences. Amplified products were purified with Ampure magnetic bead clean-up system (Agencourt, Beverly, MA). Big Dye Terminator Version 3.1 sequencing mastermix was used with the forward PCR primer and reactions were purified with CleanSEQ magnetic clean-up system (Agencourt). Sequencing was performed in duplicate on an ABI 3730 sequencer and data analysis was carried out using Sequence Scanner software (Applied Biosystems, Foster City, CA). The percentage methylation at each CpG was determined by dividing the cytosine peak by the combined heights of the cytosine and thymine peaks as described previously [22].

mRNA expression analysis

Extracted RNA was converted to cDNA using Superscript II (Invitrogen, Carlsbad, CA). Real time PCR was performed using an ABI 7500 real time PCR machine and SYBR green mastermix (Applied Biosystems) and primers listed in Additional File 1: Table T1. Reactions were carried out in triplicate and the fold change in expression was normalised to the β-actin housekeeping gene using the $2^{-\Delta\Delta Ct}$ method. For the purpose of calculating fold changes in expression, genes with no detectable expression were assigned a Ct value of 40. Standard deviations were calculated and a T-test was employed to compare expression levels in drug treated cells against untreated cells. *P*-values less than 0.05 were considered to be statistically significant.

Ethical Approvals

This study was deemed exempt from ethics approval from the University of Newcastle, and consent was not required due to use of cell lines.

Results

Global Methylation levels

Quantitative HPLC indicated that global methylation was lower in all CRC cell lines in comparison to the fibroblast cell line, with a difference of at least 2% in total methylated cytosine (Figure 1). Whilst all cancer cell lines displayed hypomethylation, there were differences in global

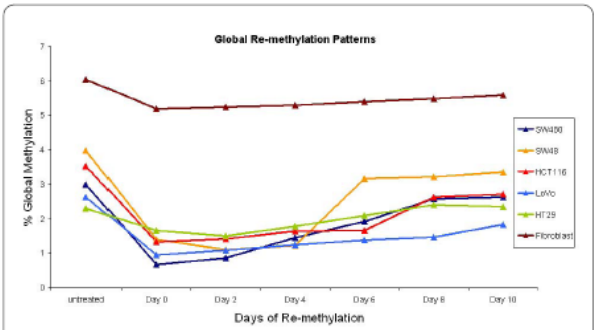

Figure 1 Global re-methylation in cell lines. All cells exhibited demethylation after 5-aza-dC treatment, and re-methylation occurred over the 10 days of drug free growth.

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methylation levels indicating these cells exhibit unique methylation profiles. The cancer cell line HT29 displayed the largest difference in methylation levels compared to the control fibroblast cell line with a total methylated cytosine content difference of 3.75%.

Global methylation response to 5-aza-dC and re-methylation

Following treatment of cells with 5-aza-dC over 72 hours, there was a substantial decrease of genomic DNA methylation. The decrease in global methylation in the SW48, SW480, HCT116 and LoVo cell lines was greater than 50%, whilst the decrease in fibroblast and HT29 cell lines were not as extensive (Figure 1). Nonetheless, all treated cells had significantly lower levels of methylation compared to untreated cells ($p < 0.01$). By the tenth day of drug free growth, global methylation levels approached those observed prior to drug treatment, and the HT29 cell line had reached pre-treatment levels by Day 8. This observation suggests there is remodelling of the chromatin state. Cytotoxicity was monitored and found to be elevated following drug treatment, which subsequently receded as growth was continued in drug-free media (Additional File 2: Figure S2). Similarly, an increase in apoptosis was induced by 5-aza-dC exposure, which gradually diminished during the drug free growth period (Table 1).

Global methylation response to combined 5-aza-dC and TSA and re-methylation

Combined TSA and 5-aza-dC treatment was performed on the HCT116 cell line to observe whether histone acetylation would influence the process of DNA re-methylation. Measurement of genomic methyl-cytosine levels revealed no synergistic effect of combined TSA and 5-aza-dC treatment on the de-methylation and no influence of acetylated histones on the re-methylation process in these cells (Table 2).

Gene-expression analysis

Numerous silenced genes were reactivated after 72 h treatment with 5-aza-dC in each cell line as determined by Illumina expression microarrays. In the cancer cell lines, eight genes were commonly reactivated (Table 3) and a greater number of these remained expressed 10 d post treatment, whilst more short term reactivation was observed in the fibroblasts (Table 4). Hyper-acetylation induced by TSA in HCT116 cells resulted in an increased number of long term and fewer short term reactivated genes compared with 5-aza-dC treatment alone. Of the 511 genes temporarily reactivated with 5-aza-dC in HCT116 cells, 165 were re-expressed in enduring manner when also subjected to 5-aza-dC and TSA combination treatment, suggesting a role of histone acetylation in long term reactivation.

Differences were observed in the resulting level of expression of reactivated genes between cell lines in the days following removal of 5-aza-dC. Filtering of the data allowed categorization of two groups of reactivated genes; those that remained highly expressed 10 d post treatment, and those which reverted to an inactive or lowly expressed state (Figure 2). Lists of genes which were expressed according to these opposing groups in two different cells lines were generated. Genes associated with a CpG island were randomly selected for bisulphite sequencing analysis to allow comparisons between long and short term reactivated genes.

Table 1: Cell Death induced by 5-aza-dC and during recovery period.

Cell Line	Time Point	Viable (%)	Necrotic (%)	Apoptotic (%)
HCT116	Untreated	94.6	5.2	0.2
	5-aza-dC treated	94.0	5.5	0.5
	4 d post treatment	89.8	9.9	0.3
	10 d post treatment	94.0	5.6	0.4
SW480	Untreated	99.8	0.1	0.1
	5-aza-dC treated	80.3	14.2	5.5
	4 d post treatment	90.5	8.7	1.4
	10 d post treatment	92.6	6.2	1.2
LoVo	Untreated	78.4	9.4	12.2
	5-aza-dC treated	67.2	7.0	25.8
	4 d post treatment	67.0	10.7	22.3
	10 d post treatment	77.6	11.3	11.1

Necrosis and cells undergoing apoptosis increased after 5-aza-dC exposure. The number of necrotic cells remained elevated ten days post treatment in SW480 and LoVo whilst apoptosis was decreasing in all cell lines ten days post treatment.

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Table 2: Genomic methylation of cells treated with 5-aza-dC alone and in combination with TSA.

Time after 5-aza-dC	5-aza-dC & TSA	5-aza-dC alone	p-value
day 0	1.52%	1.18%	0.06
day 2	1.55%	1.52%	0.82
day 4	2.29%	2.32%	0.60
day 6	2.29%	2.37%	0.14
day 8	2.31%	2.38%	0.31
day 10	2.41%	2.39%	0.76

Global methylation levels decreased with 5-aza-dC treatment and were gradually restored. T-test p-values indicate there is no synergistic effect caused by histone acetylation in the demethylation of genomic DNA.

Gene methylation and expression in response to 5-aza-dC

Bisulfite sequencing of seven individual gene promoter regions revealed that de-methylation and re-methylation changes observed at the genome-wide level were not reflected in CpG Island (CGI) methylation levels of the seven genes examined. Global levels decreased by over 50% in some cell lines, however the reduction of methylation at specific gene CGIs was significantly less. The highest de-methylation observed at a specific gene was ~25% at the *RNF113B* CGI in the HT29 (See Additional File 3: Figure S3), SW480 and HCT116 cell lines.

Up to 400 bp of sequence data was analysed by bisulfite sequencing of the CGIs surrounding the Transcription Start Site and where possible, the ATG start codon of the respective gene. Several genes revealed the presence of hypomethylated cytosines within an otherwise hypermethylated CGI, and frequently the hypomethylated cytosines would lie in close proximity to the TSS. Genes demonstrating this feature were the *MAGEA3* (NM_005362.3) (Figure 3), *CDO1* (NM_001801.2) and *HSPC105* (NM_145168.2) in SW480 cells, *MAGEA3* and *CDO1* in HT29 cells (Additional File 4: Figure S4) and *MLH1* (NM_000249.2) in SW48 cells (Additional File 5: Figure S5). These genes all appeared to be epigenetically silenced in untreated cells and were reactivated and highly expressed 10 days after 5-aza-dC removal.

Partial methylation was detected in the CGI of *MAGEA3* and *HSPC105* genes that were actively transcribed in untreated cells however no methylation was detected in the CGI associated with the beta-actin housekeeping gene in any of the cell lines tested. The *MAGEA3* and *HSPC105* genes in HCT116 and HT29 cells respectively, were both expressed and with CGI methylation of ~50-60% suggesting only one allele was hypermethylated.

HCT116 and SW48 cells are of particular interest since the hypermethylation of the promoter regions of *CDKN2A* (NM_000077.3) and *MLH1* respectively are considered to result in the repression of these genes' expression. In the HCT116 cells, the *CDKN2A* CGI displayed a region of 50% methylation in the vicinity of the TSS that spanned at least 100 bp (Additional File 6: Figure S6). Consequently, *CDKN2A* expression was detected at all times points in these cells. With the exception of SW48, *MLH1* was expressed in all cells, and no methylation was detected in MLH1 CGI. After 10 days of drug free growth, *MLH1* was still expressed in SW48 cells, but

Table 3: Commonly reactivated genes in 5-aza-dC treated colorectal cancer cells

Accession No.	Gene Name
NM_203339.1	Clusterin
NM_176791.3	Gametocyte specific factor 1-like
NM_173357.2	Synovial sarcoma, × breakpoint 6 (Pseudogene)
NM_144701.2	Interleukin-23 receptor
NM_080618.2	CCCTC-binding factor (zinc finger protein)-like
NM_032598.3	Spermatogenesis associated 22
NM_012253.2	Transkelotase-like 1
NM_006001.1	Tubulin alpha 3C

Eight genes were commonly reactivated across the five cell lines exposed to 5-aza-dC for 72 h.

Table 4: Expression patterns of reactivated genes.

Cell Line	Short Term	Long Term	Other
Fibroblasts	626	453	356
HCT116	511	702	244
HCT116 + TSA	354	943	134
SW480	383	650	246
SW48	525	778	248
HT29	551	670	257
LoVo	1116	597	185

Short term genes were transiently reactivated with 5-aza-dC whilst long term genes were still expressed 10 d post treatment. Other refers to any pattern other than short or long term re-expression.

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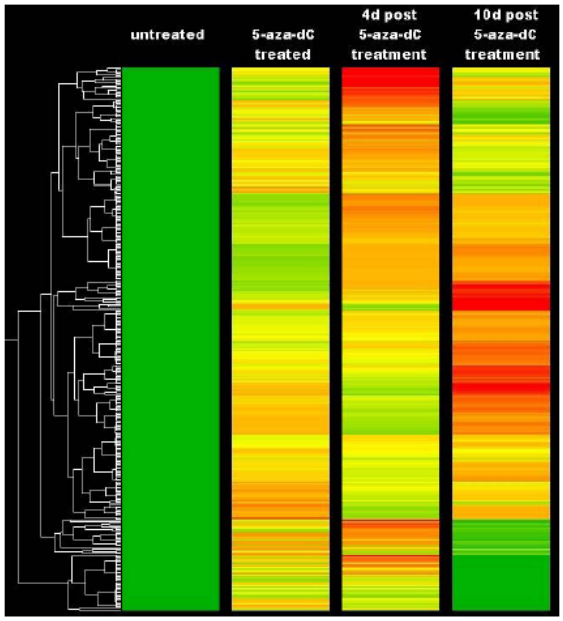

Figure 2 Selection of genes for bisulphite sequencing analysis. Shown in the heatmap are the genes reactivated in the SW480 cell line. Filters were applied to select silenced genes that were reactivated upon 5-aza-dC treatment. Genes demonstrating differential expression between cell lines were chosen for further analysis.

this did not correlate with the methylation status of the *MLH1* CpG island, which was hypermethylated.

While some genes were still highly expressed 10 days after 5-aza-dC treatment, there were a group of genes which were temporarily up-regulated or re-expressed but were expressed at low levels after 10 days of drug free growth. Genes in this category that were studied include

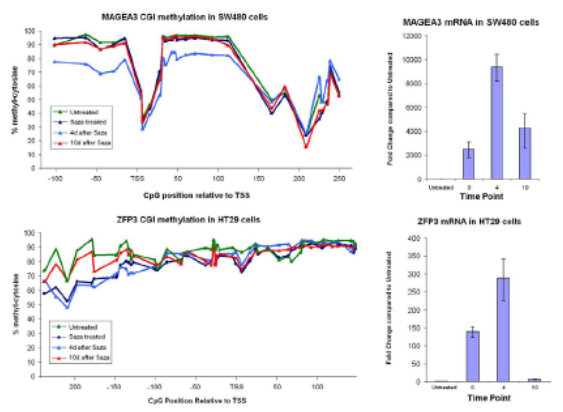

Figure 3 MAGEA3 and ZFP3 CGI methylation and expression with 5-aza-dC treatment. Figure 3A illustrates hypomethylated cytosines at the TSS in otherwise hypermethylated CpG island and the corresponding expression (B). Figure C and D shows CGI hyper-methylation and expression of the temporarily reactivated ZFP3 gene in HT29 cells.

the *ZFP3* (NM_153018.1) (Figure 3) and *RNF113B* (NM_178861.3) genes in the HT29 cell line. De-methylation was observed at the *RNF113B* CGI after 5-aza-dC exposure and this level returned to normal levels 10 days after removal of the 5-aza-dC. Genes classified as temporarily reactivated by 5-aza-dC had uniform levels of hypermethylation, unlike those classified as 'permanently' reactivated which carried hypomethylated CpG sites.

Discussion and Conclusions

The involvement of epigenetic factors, particularly DNA methylation on the regulation of gene expression has been recognised for quite some time, however it is a process not totally understood. The events which underlie genomic hypomethylation and hypermethylation of tumour suppressor genes in malignant cells and why these types of genes are targeted remain unresolved. HPLC analysis of genomic DNA was performed to assess the level of total methylation content present in several types of CRC cells. Genome wide hypomethylation was observed in all the CRC cell lines analysed when compared with the fibroblast cell line, and the lower levels of global methylation in the LoVo and HT29 cell lines may contribute to the genomic instability observed in these cell lines. This finding is consistent with previous reports that malignant cells have lower levels of genomic DNA methylation when compared with healthy tissues [23,24]. The de-methylation observed on a global level did not correlate with that found at specific CGIs, and consequently, expression was observed from genes associated with a hypermethylated promoter. The discrepancy between global and specific gene methylation levels can be explained by the reduced methylation level of Alu elements and LINE methylation. Yang and colleagues [25] demonstrated Alu elements and LINE methylation is reduced by 16% and 60% respectively in colorectal cancer cell lines treated for 72 h with 5-aza-dC. Repetitive regions are likely to fall within compacted heterochromatin where methyltransferase access to the DNA is limited and as a result these sequences become more readily hypomethylated. Furthermore, methylation reductions in a combination of transposable elements [5], satellite repeats [26] and other methylated GC rich areas of the genome which do not form *bona fide* CpG islands [27] may also contribute to this difference.

Global methylation levels and response to 5-aza-dC and TSA

In all cell lines studied significant genome-wide de-methylation was observed, and a greater than 50% reduction was observed in SW48, SW480 and HCT116 cells, indicating significant DNA de-methylation. Amongst the cancer cells, higher pre-treatment levels of global methylation appeared to correlate with a larger decrease in

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global methylation levels. Even with exposure to a high concentration of 5-aza-dC, global methylation levels following treatment averaged ~1.2% in the colorectal cancer cell lines. This may be interpreted to suggest that if total methylation levels fall below this point, cell death may be induced either through de-methylation, or toxicity from excessive DNMT binding to DNA. Further analysis of DNA with genome wide methylation arrays might reveal regions that are common to all cell lines which are resistant to DNA de-methylation induced by 5-aza-dC. This possibility was not examined in the current study.

By the tenth day of drug free growth, the global methylation of all cell lines had increased and was nearing pre-treatment levels. The rate of re-methylation was steady in the SW480, LoVo, HT29 and fibroblast cell lines, whilst periods of rapid re-methylation were observed in HCT116 and SW48 cell lines (Figure 1). Despite genomic methylation increasing, the rate was reduced dramatically after this point which may suggest levels have, or could, plateau below the original level. This scenario could be indicative of an altered pattern of gene expression following 5-aza-dC treatment. The HCT116 and SW48 cell lines are known to harbour epigenetic changes, and were among the most affected by 5-aza-dC which may suggest regulation of DNA methylation in these cell lines is different from the others studied.

Continual exposure of the HCT116 cells to Trichostatin A was performed in combination with regular 5-aza-dC treatment to investigate whether altering histone acetylation and chromatin conformation could affect the DNA methylation process and subsequent gene expression patterns. TSA is an inhibitor of Histone Deacetylase and leads to histone hyper-acetylation. Histone acetylation is associated with regions of active chromatin [28] and has been shown to assist the binding of a the TFIIIA transcription factor to chromatin templates [29]. With this knowledge, an investigation into whether global de-methylation could be enhanced and if re-methylation could be restricted by histone hyper-acetylation was undertaken. Our results show that at a genome wide level, TSA did not enhance the de-methylation process in HCT116 cells, and continual exposure to TSA for ten days did not significantly alter the re-methylation process (Table 2) in the HCT116 cell line. The p-value of 0.06 indicates the largest difference is at the d0 time point, however at this time the TSA treated cell line had a greater level of global methylation, and therefore did not enhance de-methylation. Based on these observations, DNA methylation is not hindered by histone acetylation. This notion does not conflict with previous findings that 5-aza-dC and TSA have a synergistic effect on gene expression [17,30] rather it indicates methyltransferase enzymes are not deterred from hyper-acetylated DNA.

Expression arrays were conducted with the aim of identifying reactivated genes that were differentially expressed between the cell lines and could be subjected to bisulfite sequencing analysis. In addition, the influence of TSA on expression patterns could be observed. Combined treatment of TSA with 5-aza-dC did not cause an increase in the number of reactivated genes which is in accordance with its minor influence on genomic methylation levels. Again, this does not conflict with previous reports of a synergistic effect of the two drugs, but indicates DNA methylation plays a greater role than histone acetylation in reactivating silenced genes on a genome wide level [20]. The synergistic effect of TSA was observed with prolonged expression of 165 genes deemed temporarily reactivated with 5-aza-dC alone, implicating TSA treatment as a valuable tool for maintaining expression of genes reactivated with 5-aza-dC.

The combined use of 5-aza-dC and TSA may be advantageous in overcoming poor outcomes in tumour types that do not respond to 5-aza-dC alone. A 'maintenance' administration of TSA following a 5-aza-dC treatment cycle may assist with prolonged gene expression without the cytotoxic effects of 5-aza-dC. Furthermore, identification of the pre-existing methylation patterns at genes targeted for reactivation could determine whether that gene will respond to treatment and whether a particular patient is suitable for this type of therapy.

Bisulphite sequencing of CpG islands

Bisulphite conversion of DNA followed by PCR and direct sequencing across a CGI permits quantification of the methylation at individual CpG sites and allows for the establishment of a methylation profile of CpG islands. The genes studied displayed varying levels of responsiveness to 5-aza-dC treatment, as observed by a decreased CGI methylation, however the decrease was not consistent with that observed on a genome wide level. The largest decrease in gene specific methylation was in the *RNF113B* gene. After treatment, CGI methylation levels dropped by over 20% in the HT29 cell line with an associated increase in gene expression. By the tenth day of drug-free growth the CGI methylation returned to pre-treatment levels, which correlated with the return of normal levels of gene expression.

Sequencing of CGIs allowed the detection of several instances where a small cluster of cytosines were hypomethylated amongst an otherwise hypermethylated CpG island in a non-expressed gene. These hypomethylated cytosines appear at a CpG site adjacent to the TSS of a gene, as seen in the *MAGEA3* CGI in SW480 cells. Upon culturing the cells for a further four and ten days in drug free media, these cells were found to still be expressing the previously silenced *MAGEA3* gene, suggesting the

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transcriptional status of this gene had been permanently reversed. In comparison, the HCT116 cell line expressed these two genes constitutively. The only common methylation pattern amongst the two cell lines was <50% methylation at the CpG sites 1 bp upstream and 10 bp downstream of the TSS in the *MAGEA3* gene. This kind of reactivation was also observed in *CDO1*, and *HSPC105* genes in SW480 cells which also carried hypomethylated CpG sites.

To investigate the possibility that the enduring reactivation of genes was due to continued cytotoxicity or apoptosis, we monitored these levels over the corresponding time period. Both cytotoxicity and apoptosis levels were elevated by 5-aza-dC, but decreased when the drug was removed. This indicates gene expression variation is a result of changes in genomic methylation rather than activation of apoptotic pathways. The fraction of necrotic cells remained higher than in untreated cells which are likely to represent reactivated genes that orchestrate cell death or cells that died in the time prior to the measurement of apoptosis.

Treatment of cells with 5-aza-dC caused the reactivation of numerous genes, although de-methylation within the CGI of specific genes did not correlate with genomic levels (as discussed earlier) or transcription levels. Consequently we observed that 5-aza-dC induced expression can be driven from a largely methylated promoter with localised demethylation at the TSS. One such example is the *CDO1* gene in SW480 cells (Additional File 7: Figure S7), where ~10% de-methylation (ie 10% of all alleles) at three CpG sites caused a greater than 1000-fold up-regulation of CDO1 mRNA.

We envisage the significant increase in expression of CDO1 is inflated due to non-existent expression in untreated cells, and that modest expression is permitted due to hypomethylation in a small proportion of cells at the TSS. As methylation of surrounding CpG sites was largely unaltered, demethylation of three CpG sites in a 50 bp region surrounding the TSS is permissive of transcription, and does not require hypomethylation of the entire allele. A similar finding has been previously reported in *CDKN2A* in cervical carcinogenesis [31] and also in the *CDH1* gene in [32]. The minor demethylation of *CDH1* in conjunction with large increase of gene expression may suggest that DNA methylation does not repress transcription, and gene up-regulation could be due to either a secondary effect or the involvement of other factors which are also modified by 5-aza-dC treatment.

Clusters of CpG sites were identified in some genes that showed a region of hypomethylation, such as *MAGEA3* in HT29 cells (Additional File 4: Figure S4) and *ZFP3* in the SW480 cells (Additional File 8: Figure S8). Expression of these genes was detected before 5-aza-dC treatment, demonstrating the importance of methylation in the CpG sites around the TSS, and how a region of hypomethylation is permissive of transcription. Methylation of ~50% was detected at some CGIs including *CDKN2A* in HCT116 cells. These genes are likely to show monoallelic methylation which has been reported previously [33,34], where all expression is from one non-methylated allele.

The *MLH1* gene is silenced by methylation on both alleles in SW48 cells [35]. Following 5-aza-dC treatment, de-methylation was induced and the gene was re-expressed and by the tenth day of drug free growth, the gene was still expressed despite methylation returning to pre-treatment levels. The expression level of *MLH1* at day 10 may be due to lower methylation at a CpG site 32 bp downstream from the TSS, which is similar to the long term reactivation in the *CDO1*, *MAGEA3* and *HSPC105* genes in SW480 cells. The analysis undertaken suggests that genes thought to be under control of epigenetic modifications such as *CDKN2A* and *MLH1* were not shown to have significantly altered patterns of CpG methylation following 5-aza-dC treatment, although an increase in expression was observed.

In non-expressed genes, the identification of regions of hypomethylated cytosine in a generally hypermethylated CpG island raises the question of how these cytosines are maintained in a hypomethylated state. As the region of hypomethylated cytosines in these genes is less than 146 bp - the length of DNA associated with a nucleosome, it would suggest in this scenario at least, an absent nucleosome is not a factor in assisting transcription as previously described [36]. It would appear 5-aza-dC can induce an irregular nucleosomal conformation that permits expression from methylated genes. It is also possible that 5-aza-dC reduces repressive histone tail marks or initiates the gain of active marks in the area surrounding the hypomethylated cytosine following 5-aza-dC exposure. The methylation of Histone H3 Lysine 9 is a modification associated with inactive chromatin and has been shown to be rapidly lost with 5-aza-dC treatment [37]. It is possible that a loss of H3K9 methylation may have been induced by 5-aza-dC in the current study, which may allow binding of transcriptional proteins, which in turn enhance transcriptional reactivation.

Hypomethylated CpG sites which exert control on gene expression may have implications for methods such as methylation-specific PCR and array technologies which rely on the methylation status of a small number of CpG sites in order to determine a given genes methylation status. The observation that a single or small group of CpG sites could affect expression may have greater implications should a polymorphism exist at the site. A polymorphism at the CG dinucleotide will deny methyl-group attachment which would be advantageous to individuals

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with that change by conferring a protective effect against epigenetic inactivation, particularly in genes such as *MLH1*.

Our results show that 5-aza-dC induces gene expression, but is not necessarily dependant on DNA de-methylation. The pre-existing level of methylation surrounding the transcriptional start site of a gene appears important in long term reactivation. This was demonstrated as the transcriptional status of silenced genes could be reversed with 5-aza-dC and for up to ten days after its removal in genes with hypomethylated cytosines despite minimal CpG de-methylation. In CGIs which exhibited a uniform level of hypermethylation, transcription could be induced for a short term only. The acetylation of histones was not found to alter the de-methylation or re-methylation process and was therefore not expected to cause a change in the gene expression profile. Although constitutively expressed genes demonstrate hypomethylation, 5-aza-dC treatment was found to force expression from genes with hypermethylated CGIs, suggesting that 5-aza-dC is capable of influencing other factors involved with gene expression, such as proteins with a methyl-binding domain or histone modifications.

Additional material

Additional file 1 Table S1. Primer sequences used in Bisulfite PCR/ Sequencing and qPCR.

Additional file 2 Figure S2. 5-aza-dC induced cytotoxicity levels and recovery. Cytotoxicity was elevated immediately following treatment. By day 10 of the recovery period these levels had subsided to at least half of the initial value.

Additional file 3 Figure S3. RNF113B methylation in HT29 cells. The RNF113B gene is methylated (A) and lowly expressed (B) in HT29 cells. 5-aza-dC induces de-methylation of up to 30% which corresponds with an increase in expression of the gene. Methylation of the RNF113B CGI and expression at day 10 approach levels observed in untreated cells.

Additional file 4 Figure S4. MAGEA3 and CDO1B CGI methylation in HT29 cells. Both of these CGIs demonstrate hypomethylation at the TSS (A and C). Following 5-aza-dC treatment both genes were still expressed after 10 days of drug free growth (B and D).

Additional file 5 Figure S5. MLH1 CGI methylation in SW48 cells. The methylation of SW48 does not change dramatically with 5-aza-dC treatment (A), but expression is reactivated and remains high after ten days of drug free growth (B).

Additional file 6 Figure S6. CDKN2A CGI methylation in HCT116 cells. Methylation of the CDKN2A CGI is approximately 50% surrounding the TSS (A). Expression is detected in untreated cells and becomes up-regulated after 5-aza-dC treatment (B).

Additional file 7 Figure S7. CDO1 CGI methylation in SW480 cells. A decrease of ~10% of promter methylation in the CDO1 promoter region results in an 1000 fold increase in expression.

Additional file 8 Figure S8. ZFP3 CGI methylation in SW480 cells. A cluster of hypomethylated cytosines are present at the TSS but a greater level of methylation is observed adjacent to this region. Expression is up-regulated and remains high after ten days of drug free growth.

Abbreviations

CGI: CpG Island; CRC: Colorectal Cancer; DNA: deoxyribonucleic acid; 5-aza-dC: 5-aza-2'-deoxycytidine; HPLC: High Performance Liquid Chromatography; MeCP: Methyl-CpG binding Protein; PBS: Phosphate Buffered Saline; PCR: Polymerase Chain Reaction; RNA: ribonucleic acid; TSA: Trichostatin A; TSS: Transcription Start Site.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DM performed the HPLC, microarray, bisulfite sequencing assays, analysis and drafted the manuscript. KTK performed the cytotoxicity and cell survival assays. RJS conceived the study, and participated in its design and co-ordination. All authors have read and approved the final manuscript.

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References

1. Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A, Reinberg D: **Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation.** *Genes Dev* 1999, **13(15):**1924-1935.
2. Meehan RR, Lewis JD, Bird AP: **Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA.** *Nucleic Acids Res* 1992, **20(19):**5085-5092.
3. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, *et al.*: **Initial sequencing and analysis of the human genome.** *Nature* 2001, **409(6822):**860-921.
4. Wong LH, Choo KH: **Evolutionary dynamics of transposable elements at the centromere.** *Trends Genet* 2004, **20(12):**611-616.
5. Yoder JA, Walsh CP, Bestor TH: **Cytosine methylation and the ecology of intragenomic parasites.** *Trends Genet* 1997, **13(8):**335-340.
6. Jubb AM, Bell SM, Quirke P: **Methylation and colorectal cancer.** *J Pathol* 2001, **195(1):**111-134.
7. Feinberg AP, Vogelstein B: **Hypomethylation distinguishes genes of some human cancers from their normal counterparts.** *Nature* 1983, **301(5895):**89-92.
8. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP: **Hypomethylation of DNA from benign and malignant human colon neoplasms.** *Science* 1985, **228(4696):**187-190.
9. Kim HC, Roh SA, Ga IH, Kim JS, Yu CS, Kim JC: **CpG island methylation as an early event during adenoma progression in carcinogenesis of sporadic colorectal cancer.** *J Gastroenterol Hepatol* 2005, **20(12):**1920-1926.
10. Matsuzaki K, Deng G, Tanaka H, Kakar S, Miura S, Kim YS: **The relationship between global methylation level, loss of heterozygosity, and microsatellite instability in sporadic colorectal cancer.** *Clin Cancer Res* 2005, **11(24 Pt 1):**8564-8569.
11. Liu L, Wylie RC, Andrews LG, Tollefsbol TO: **Aging, cancer and nutrition: the DNA methylation connection.** *Mech Ageing Dev* 2003, **124(10-12):**989-998.
12. Michalowsky LA, Jones PA: **Differential nuclear protein binding to 5-azacytosine-containing DNA as a potential mechanism for 5-aza-2'-deoxycytidine resistance.** *Mol Cell Biol* 1987, **7(9):**3076-3083.
13. Balch C, Yan P, Craft T, Young S, Skalnik DG, Huang TH, Nephew KP: **Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer.** *Mol Cancer Ther* 2005, **4(10):**1505-1514.
14. Tsuji N, Kobayashi M, Nagashima K, Wakisaka Y, Koizumi K: **A new antifungal antibiotic, trichostatin.** *J Antibiot (Tokyo)* 1976, **29(1):**1-6.

15. Yoshida M, Kijima M, Akita M, Beppu T: **Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A.** *J Biol Chem* 1990, **265(28)**:17174-17179.
16. McGhee JD, Felsenfeld G: **Nucleosome structure.** *Annu Rev Biochem* 1980, **49**:1115-1156.
17. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB: **Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer.** *Nat Genet* 1999, **21(1)**:103-107.
18. Wu Y, Alvarez M, Slamon DJ, Koeffler P, Vadgama JV: **Caspase 8 and maspin are downregulated in breast cancer cells due to CpG site promoter methylation.** *BMC Cancer* **10(32)**:32.
19. Ooki A, Yamashita K, Kikuchi S, Sakuramoto S, Katada N, Kokubo K, Kobayashi H, Kim MS, Sidransky D, Watanabe M: **Potential utility of HOP homeobox gene promoter methylation as a marker of tumor aggressiveness in gastric cancer.** *Oncogene* 2010:15.
20. Dannenberg LO, Edenberg HJ: **Epigenetics of gene expression in human hepatoma cells: expression profiling the response to inhibition of DNA methylation and histone deacetylation.** *BMC Genomics* 2006, **7**:181.
21. Flatmark K, Nome RV, Folkvord S, Bratland A, Rasmussen H, Ellefsen MS, Fodstad O, Ree AH: **Radiosensitization of colorectal carcinoma cell lines by histone deacetylase inhibition.** *Radiat Oncol* 2006, **1**:25.
22. Deng G, Chen A, Hong J, Chae HS, Kim YS: **Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression.** *Cancer Res* 1999, **59(9)**:2029-2033.
23. Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M: **Reduced genomic 5-methylcytosine content in human colonic neoplasia.** *Cancer Res* 1988, **48(5)**:1159-1161.
24. Cui H, Onyango P, Brandenburg S, Wu Y, Hsieh CL, Feinberg AP: **Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2.** *Cancer Res* 2002, **62(22)**:6442-6446.
25. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP: **A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements.** *Nucleic Acids Res* 2004, **32(3)**:e38.
26. Mund C, Hackanson B, Stresemann C, Lubbert M, Lyko F: **Characterization of DNA demethylation effects induced by 5-Aza-2'-deoxycytidine in patients with myelodysplastic syndrome.** *Cancer Res* 2005, **65(16)**:7086-7090.
27. Brock GJ, Charlton J, Bird A: **Densely methylated sequences that are preferentially localized at telomere-proximal regions of human chromosomes.** *Gene* 1999, **240(2)**:269-277.
28. Kornberg RD, Lorch Y: **Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome.** *Cell* 1999, **98(3)**:285-294.
29. Lee DY, Hayes JJ, Pruss D, Wolffe AP: **A positive role for histone acetylation in transcription factor access to nucleosomal DNA.** *Cell* 1993, **72(1)**:73-84.
30. Meng CF, Zhu XJ, Peng G, Dai DQ: **Re-expression of methylation-induced tumor suppressor gene silencing is associated with the state of histone modification in gastric cancer cell lines.** *World J Gastroenterol* 2007, **13(46)**:6166-6171.
31. Nehls K, Vinokurova S, Schmidt D, Kommoss F, Reuschenbach M, Kisseljov F, Einenkel J, von Knebel Doeberitz M, Wentzensen N: **p16 methylation does not affect protein expression in cervical carcinogenesis.** *Eur J Cancer* 2008, **44(16)**:2496-2505.
32. Tachibana K, Takeda K, Shiraishi M: **5-Aza-2'-deoxycytidine reactivates the CDH1 gene without influencing the methylation of the entire CpG island or histone modification in a human cancer cell line.** *Proc Japan Acad* 2004, **80(7)**:342-348.
33. Myohanen SK, Baylin SB, Herman JG: **Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia.** *Cancer Res* 1998, **58(4)**:591-593.
34. Mund C, Beier V, Bewerunge P, Dahms M, Lyko F, Hoheisel JD: **Array-based analysis of genomic DNA methylation patterns of the tumour suppressor gene p16INK4A promoter in colon carcinoma cell lines.** *Nucleic Acids Res* 2005, **33(8)**:e73.
35. Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, MacLeod AR: **DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells.** *Nat Genet* 2003, **33(1)**:61-65.
36. Chavez S, Beato M: **Nucleosome-mediated synergism between transcription factors on the mouse mammary tumor virus promoter.** *Proc Natl Acad Sci USA* 1997, **94(7)**:2885-2890.
37. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, Jones PA: **Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine.** *Cancer Res* 2002, **62(22)**:6456-6461.

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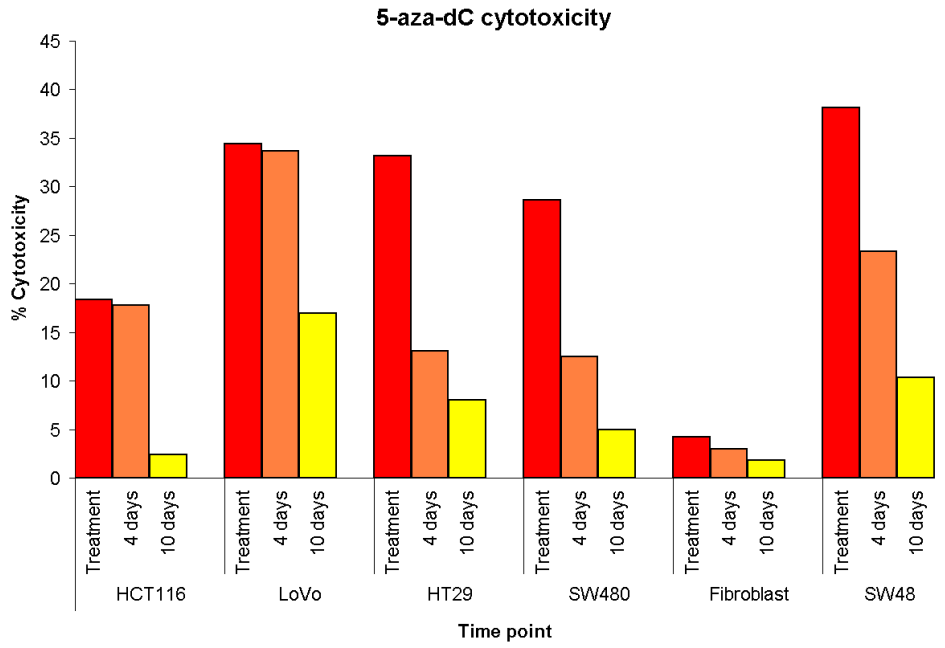
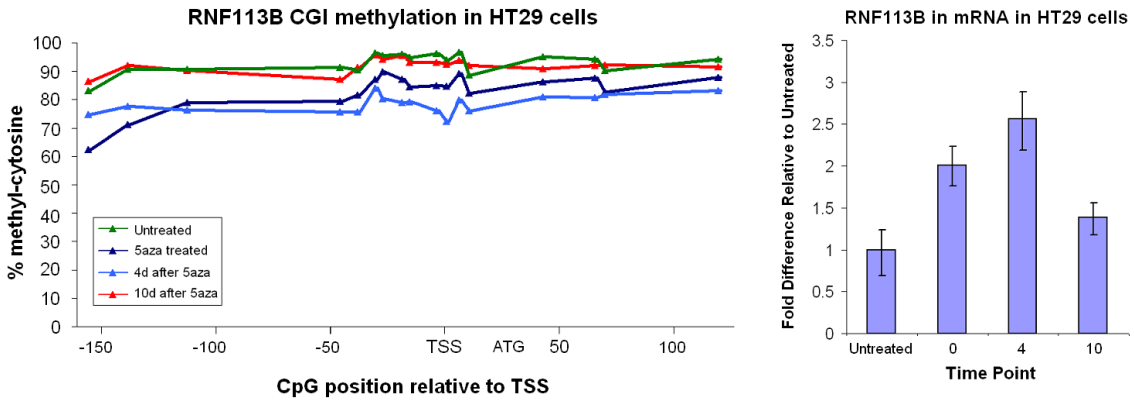
Additional File 1: Table S1 – Primer sequences used in bisulphite PCR / Sequencing and qPCR.

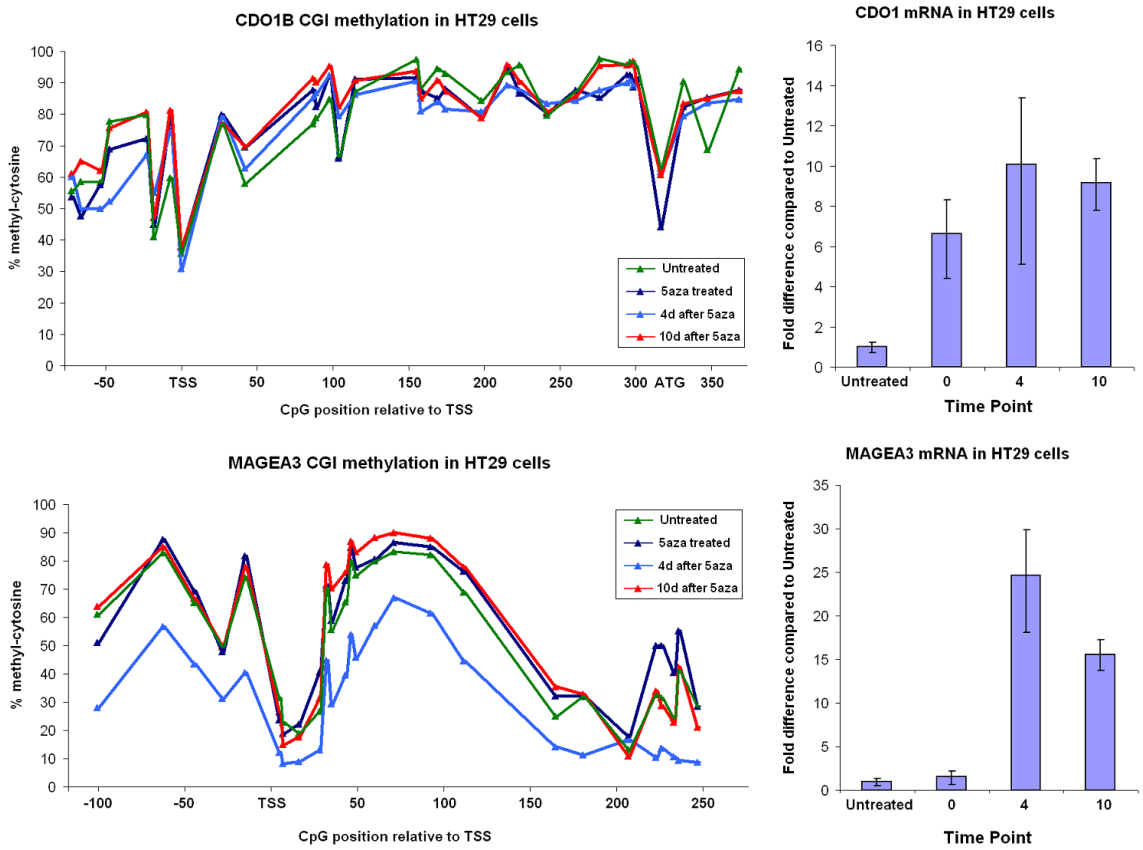
Gene (Accession Number)	**Bisulfite PCR and Sequencing (5'-3')**	**qPCR (5'-3')**
GAPDH **(NM_002046.3)**	F: GTTGGGATTGGTTGAGTT	n/a
	R: CCAAACCTCCATACCCAAC	
ACTB **(NM_001101.2)**	n/a	F: TGTGGCATCCACGAA ACTACC
		R: ACATCTGCTGGAAGGTGGACA
CDO1 **(NM_001801.2)**	F: TTAAAGTGGGGGAGAGATTG	F: GAGGGAAAACCAGTGTGCCTAC
	R: AACCTACACCTCCTCTACATTA	R: GCTCACAGCAGGTTCCGTATG
HSPC105 **(NM_145168.2)**	F: GTGAAAGTTTAAAAGTAGATAT	F: GTGTCCTCATTACAGGAGG
	R: CATTCTAAAAAACCAAACTAC	R: GCTTTCTCTACGTCAGACAGG
MAGEA3 **(NM_005362.3)**	F: GGATTTATAGTTTTAGGAT	F: ATCTGCCAGTGGGTCTCCATT
	R: CACATTAAACTCTATCCCCAAAA	R: TCTGCTCAAGAGGCATGATGA
RNF113B **(NM_178861.3)**	F: GGTTAGGTTGGTTTTAAATTGTTGATT	F: GTGTTTCATATGTCGCCAGGCC
	R: CTAAAACCTACAACCCCTTTC	R: CGGTTGGCTGGTCACAGATG
ZFP3 **(NM_153018.1)**	F: GAGTTTTTGAGTTTAGAGTAATGT	F: CTTCGGGCAGAGTTCTGAGC
	R: CATAAACTTCAAAATCACACAAC	R: CTGAGTTCCCCCTGAAGGCC
CDKN2A **(NM_000077.3)**	F: GATTTTAGGGGTGTTAT	F: GTCGGAGGCCGATCCAGGTCATG
	R: CTCATTCCTCTTCCTTAAC	R: AGCGTGTCCAGGAAGCCCTC
MLH1 **(NM_000249.2)**	F: AGATTATTTTAGTAGAGG	F: AGCTGATGGAAAGTGTGCATACA
	R: AAAAAACCTAACTAACA	R: CGTGATCTGGGTCCCTTGA



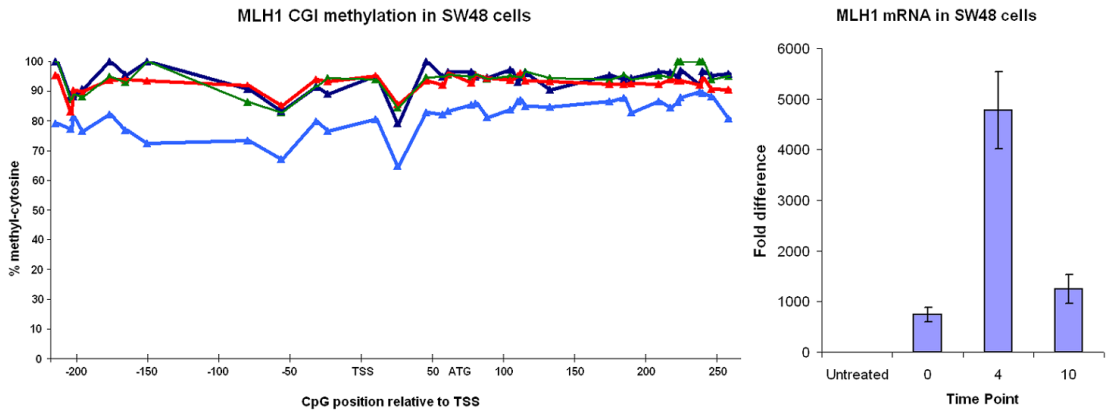
Additional File 2: Figure S2 - 5-aza-dC induced cytotoxicity levels and recovery. Cytotoxicity was elevated immediately following treatment. By day 10 of the recovery period these levels had subsided to at least half of the initial value.



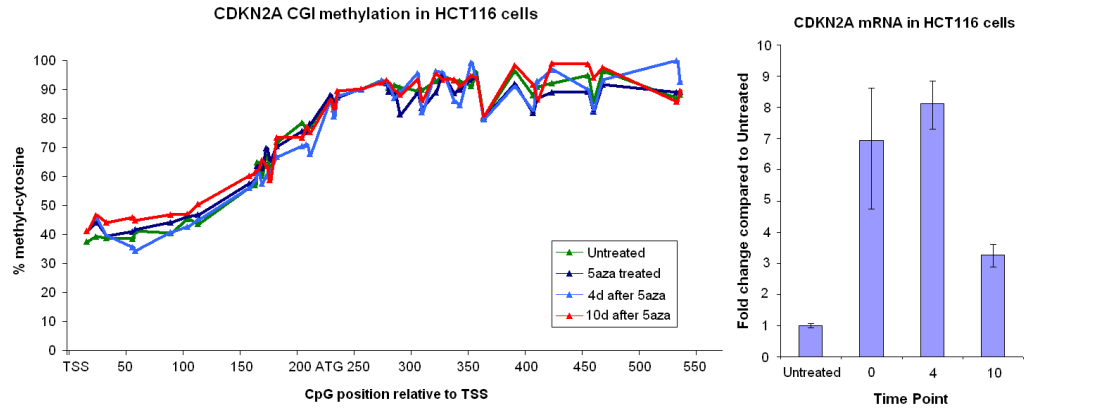
Additional File 3: Figure S3 - *RNF113B* methylation in HT29 cells. The *RNF113B* gene is methylated (A) and lowly expressed (B) in HT29 cells. 5-aza-dC induces de-methylation of up to 30% which corresponds with an increase in expression of the gene. Methylation of the *RNF113B* CGI and expression at day 10 approach levels observed in untreated cells.



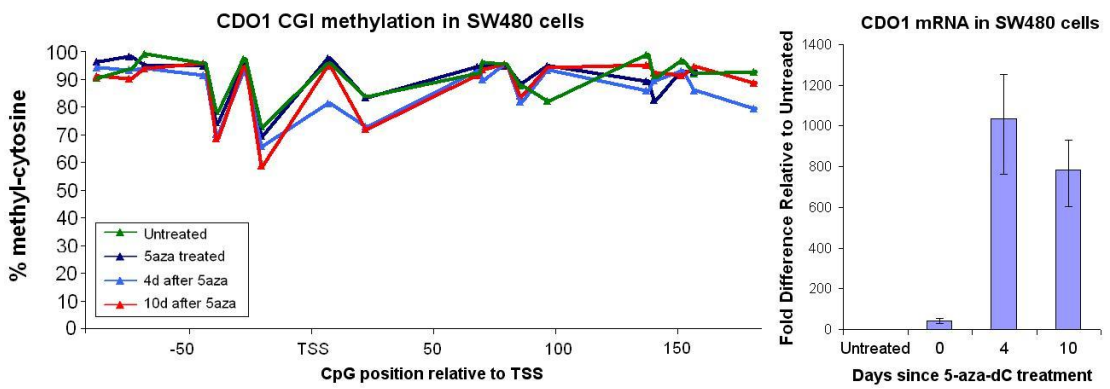
Additional File 4: Figure S4 - *MAGEA3* and *CDO1* CGI methylation in HT29 cells. Both of these CGIs demonstrate hypomethylation at the TSS (A and C). Following 5-aza-dC treatment both genes were still expressed after 10 days of drug free growth (B and D).



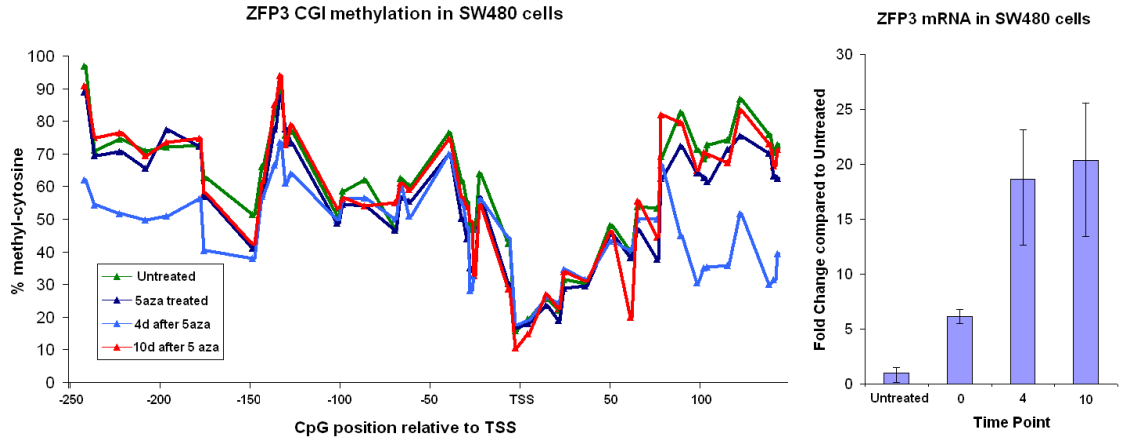
Additional File 5: Figure S5 - *MLH1* CGI methylation in SW48 cells. The methylation of SW48 does not change dramatically with 5-aza-dC treatment (A), but expression is reactivated and remains high after ten days of drug free growth (B).



Additional File 6: Figure S6 - *CDKN2A* CGI methylation in HCT116 cells. Methylation of the *CDKN2A* CGI is approximately 50% surrounding the TSS (A). Expression is detected in untreated cells and becomes up-regulated after 5-aza-dC treatment (B).



Additional File 7: Figure S7 - *CDO1* CGI methylation in SW480 cells. A decrease of ~10% of promter methylation in the *CDO1* promoter region results in a 1000-fold increase in expression.



Additional File 8: Figure S8 - *ZFP3* CGI methylation in SW480 cells. A cluster of hypomethylated cytosines are present at the TSS but a greater level of methylation is observed adjacent to this region. Expression is up-regulated and remains high after ten days of drug free growth.

Chapter 4

Molecular responses of colorectal cancer cells to 5-aza-2'-deoxycytidine

STATEMENT III

This statement explains the contribution of all authors in the article listed below:

Mossman, D. and Scott, R.J., (2009) Molecular responses of colorectal cancer cells to 5-aza-2'-deoxycytidine. Submitted to Mutagenesis and Carcinogenesis, July 2011.

Table III: Author contribution Percentage and Description of Contribution to the article listed above.

Author	Contribution (%)	Description of Contribution to Article	Signature
David Mossman	90%	Executed the study. Analysed and interpreted the data. Wrote the manuscript.	
Rodney J. Scott	10%	Designed the study, provided the concept and corrected the manuscript.	

Chapter Introduction:

Epigenetic aberrations are a common event in tumour tissues which include hypermethylation of critical genes and generalised decrease in global methylation which may also lead to the activation of proto-oncogenes. Despite the known toxicity of 5-aza-dC, the use of DNA methyltransferase inhibitors is largely ineffective against solid tumours but has given promising results in haematological disorders. In this study we analysed the gene expression patterns after treatment of 5-aza-dC on colorectal cancer cell lines to understand the molecular response in colorectal cancer cells. This study was undertaken to identify patterns of gene expression before, during and after 5-aza-dC treatment to characterise the response to drug treatment that may identify shortfalls or avenues for potential new therapies. The combined use of DNA methyltransferase and histone deacetylase inhibitors were investigated, along with a recently described re-setting of gene expression by drug treatment, where patterns of expression resemble that of a different cell type. Overall these results show that the apoptotic effect generated does not appear sufficient to kill all cells and suggest that combined therapeutic treatments against solid tumours may lead to more successful treatment strategies.

Molecular responses of colorectal cancer cells to 5-aza-2'-deoxycytidine

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Key Words: DNA methylation, 5-aza-2'-deoxycytidine, Trichostatin A, acetylation, gene expression profiling.

Running head: Molecular responses to 5-aza-dC in CRC cells

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Abstract

A wealth of evidence implicates epigenetic aberrations in virtually all forms of malignancy, yet a clear understanding of how this is established or how it can be successfully treated remains elusive. Therapies to counteract epigenetic anomalies include 5-aza-2'deoxycytidine and Trichostatin A but their effectiveness is limited to certain tumour types.

In this study we have treated colorectal cancer cell lines with both agents to examine their influence on genome wide gene expression. RNA was extracted and expression was measured with Illumina Human Ref-8 arrays.

Large-scale similarity was not detected between cell lines after 5-aza-dC exposure and expression profiles of each cell line remained similar at each time point of treatment. Furthermore, a pattern of expression resembling that of healthy epithelial cells was not induced in the cancer cell lines by 5-aza-dC treatment. Two subsets of genes were identified in each cell line that showed either transient or lasting reactivation. In HCT116 cells, co-treatment with TSA resulted in higher levels of long term reactivation compared with 5-aza-dC alone.

In terms of specific genes, a down-regulation of PRKACB was commonly identified following drug treatment, which may have pro-apoptotic downstream effects, however this mechanism does not appear to be sufficient to initiate cell death in these cells.

Our results suggest treatment with 5-aza-dC followed with maintenance Trichostatin A exposure enhances long term re-expression of genes, regardless of whether the gene is associated with a CpG island. Responses to treatment were complex and although the apoptotic pathway was not directly affected, there is evidence that genes from this pathway were altered.

Introduction

Epigenetic control of gene expression is mediated by DNA methylation and histone protein modifications. Aberrant DNA hypermethylation and histone hypo-acetylation are frequent events in malignancy which co-operatively silence critical genes. For this reason, these pathways are suitable targets for therapy against malignancy. Two classes of chemical agents exist which are capable of modifying the epigenetic status. 5-aza-2'-deoxycytidine (5-aza-dC) is a powerful inhibitor of DNA methylation that exerts its effect via the irreversible binding of DNA methyltransferase to 5-aza-dC substituted DNA, thereby depleting the cell of available methyltransferase. However, the clinical benefits of this agent are below expectations for solid tumour types and its effectiveness appears to be limited to myelodysplastic syndromes [1-3]. The second agent, Trichostatin A (TSA) is a potent inhibitor of histone de-acetylation. Whilst originally developed as an antifungal agent, TSA causes an accumulation of acetylated histones via the inhibition of histone de-acetylase [4] and results in actively expressed transcripts of affected genes [5].

These treatments appear to have varied effects on gene expression. 5-aza-dC is a well known activator of gene expression, and TSA has a similar effect but predominantly on genes not affected by hypermethylation [6, 7]. There is evidence to show that rRNA genes can be re-expressed with either 5-aza-dC or TSA [8]. In this instance, combined treatment with both agents did not result in additional re-expression, however other studies have observed a synergistic effect with 5-aza-dC and TSA [9]. Combinations of treatments may prove useful in eliciting responses with a view to a more positive outcome in particular

tumour types, but may require clear identification of the gene pathways to be targeted.

A resetting effect of gene expression profiles has previously been described in which fibroblast cells were reprogrammed to a pluripotent state with 5-aza-dC and TSA [10]. Tumour cells exposed to 5-aza-dC may also experience a resetting effect whereby they display a pattern of expression similar to that of healthy tissue. To date, the changes in genomic methylation patterns and gene expression after 5-aza-dC treatment remain poorly characterised, and the resulting expression profiles after such treatments as 5-aza-dC and TSA may reveal pathways that lead to favourable outcomes in some tumour types but not others. In this study we have used several colorectal cancer (CRC) cell lines which are known to carry many epigenetic aberrations.

We aimed to assess gene pathways that were altered after 5-aza-dC treatment to investigate the molecular response induced with drug treatment. We also sought to identify the extent of similarity between treated cancer cell lines and healthy fibroblasts, which may suggest a reversion of phenotype. To further aid the understanding of 5-aza-dC in tumour cells, we endeavoured to identify commonly affected genes which may play a role in the efficacy of 5-aza-dC in tumour cells. Characterisation of the molecular changes and effects of 5-aza-dC on colorectal tumour types may identify pathways which allow sensitisation to other cytotoxic agents and more effective treatments.

Methods

Cell Culture

All cell lines were cultured with DMEM media supplemented with 10% FCS at 37ºC and 5% CO_2. Colorectal cancer cells HCT116, SW480, SW48, LoVo and HT29 were originally obtained from the American Type Culture Collection. GM03652 fibroblasts representative of healthy epithelial cells were obtained from Coriell Cell Repository. 5-aza-dC (Sigma-Aldrich) was added at 15µM, or 10µM for LoVo and fibroblast cell lines, which have previously been used to induce maximal DNA de-methylation [11]. The extent of apoptosis and cytotoxicity induced by 5-aza-dC in these cells have also been previously reported [11]. 5-aza-dC treatment occurred over 72 h, with media replaced every 24 h. DNA and RNA were extracted from untreated cells and after 72h of 5-aza-dC exposure ('treated' time point). After 72 h of treatment, the cells were washed with PBS to remove any residual 5-aza-dC, and continued to grow for a further 10 days in drug free media. During this time, DNA and RNA were extracted after 4 and 10 days of drug free growth (d4 and d10), which represent 4 and 10 days of re-methylation. This method was then repeated in the widely studied HCT116 cells, which were continually treated with 150nM TSA (Sigma-Aldrich), with a maximum of two days between media changes. This concentration has been previously shown to cause histone acetylation in HCT116 cells [12].

Expression Analysis

Illumina Human Ref 8 Version 2 BeadChips were used to quantify changes in

version 3.0 where values for each sample were subjected to cubic spline normalisation, and the t-test error model was applied to all samples. Data were then imported to GeneSpring Software version 7.3.1 (Agilent) and normalised to the 50th percentile on a 'per chip' basis to control for intensity variations across the arrays, and a second 'per gene' normalisation was used to control for the variation in detection efficiency between gene signals. Significant changes in expression were determined with an unpaired t-test and lists of genes displaying particular patterns of expression (i.e. reactivation, down-regulation etc) were generated. Expression data obtained for day 4 after drug treatment was used as an intermediate time point to confirm long term re-expression. Significant pathways were identified using the DAVID Functional Annotation Tool and KEGG pathway [13].

Results

Characteristic features of cancerous cells include genomic DNA hypomethylation and gene specific hypermethylation, resulting in altered patterns of gene expression in comparison to healthy cells. The cancer cell lines examined here have all been previously reported to possess epigenetic aberrations which are likely to lead to abnormal gene expression patterns [14, 15]. Unsupervised cluster analysis was performed from all data points and all cell lines (Figure 1). Each cell line displays a unique expression profile, and following 5-aza-dC there is a high similarity to expression profiles from other time points of the same cell type. There is limited or no relatedness between cell lines at certain time points, such as immediately after treatment. Additionally there appears to be segregation between untreated / treated and day 4 / day 10 expression patterns which is evident across all the tested cancer cell lines, indicating sub-groups of expression patterns occur at these times.

Influence of 5-aza-dC on gene expression

To assess the extent of genome wide changes after treatment and similarity between untreated fibroblasts and treated cancer cells, the expression profiles of cancer cells were compared with those of the fibroblasts. Fibroblasts are representative of healthy epithelial cells and we aimed to identify if there was any reversion of cancer cell expression profiles to those of normal epithelial cells. The level of expression of the cancer cells (untreated, treated and d10 post treatment) was subtracted from baseline expression observed in the fibroblasts to identify different expression levels. These were then ordered according to the

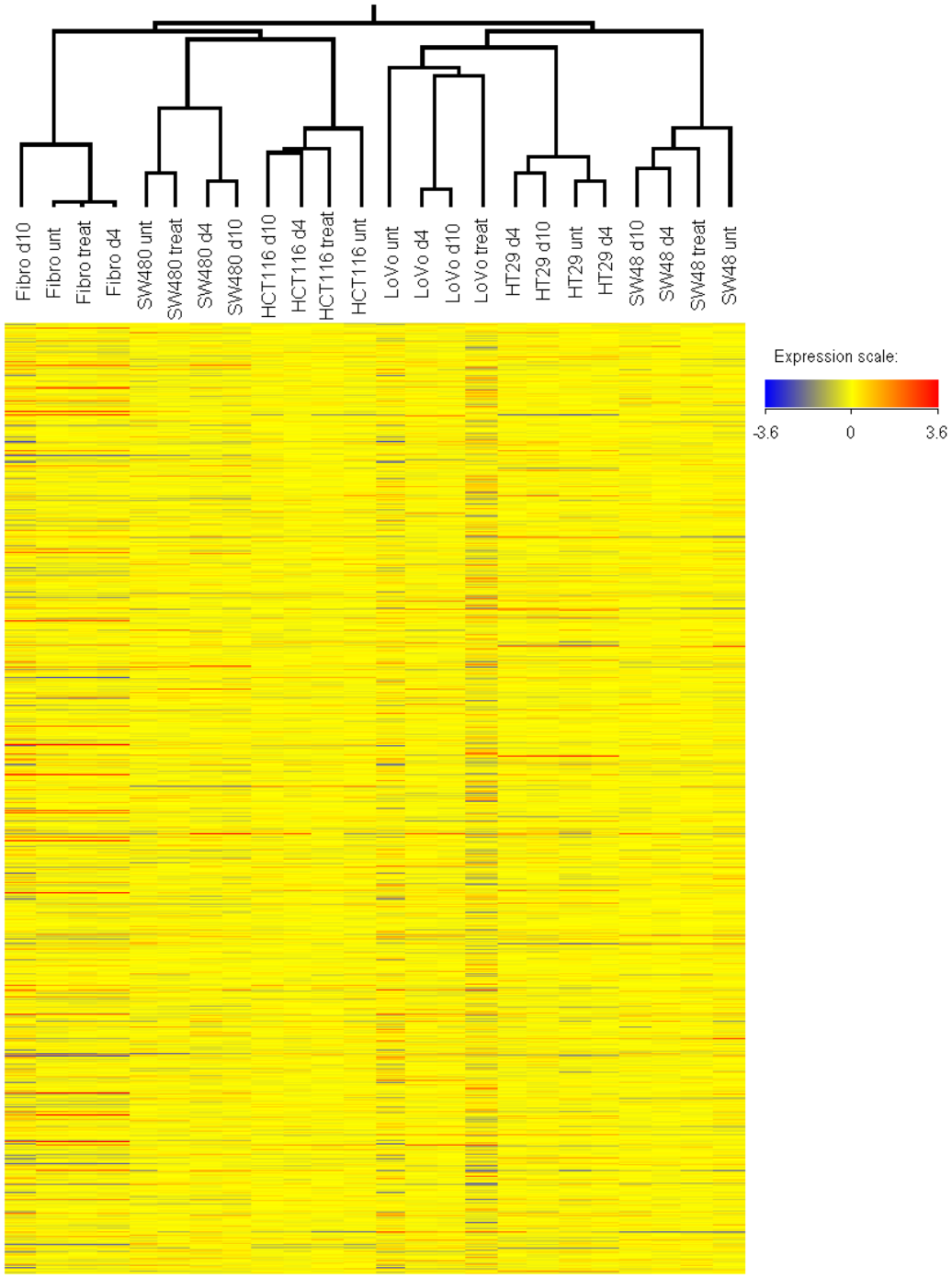

Figure 1 – Heatmap of expression levels at all time point of all cells. Differences were detected between cell lines, and expression profiles within a particular cell line were similar at all time points of treatment.

level of difference from highest to lowest in the untreated cancer cell lines. Graphs were generated to visually demonstrate the differences in expression in untreated cells and changes in expression after 72h treatment and d10 after cessation of treatment (Figure 2). A flat line would indicate 100% correlation between fibroblast and cancer cell expression. Genes at the left end of the graph in the 'A' panels are highly expressed in fibroblasts but lowly or not expressed in the cancer cell line. Genes to the right are highly expressed in cancer cell line but lowly or not expressed in the fibroblasts. Upon treatment of cancer cell lines (B panels) many genes became largely up and down-regulated as shown by downward and upward peaks respectively. By the tenth day of drug free growth (C panels), numerous genes have remained up-regulated in comparison with untreated and treated time points which is indicative of long term changes in gene expression. Large scale changes are induced such that numerous genes are reactivated or up-regulated in cancer cells to levels beyond that of fibroblast cells. Unlike the other cell lines, large changes in expression appeared to be transient in the HCT116 cell line as fewer distinct peaks were present at day 10 than after treatment. These graphs demonstrate that many small and several large changes are elicited in particular genes, but the expression profiles largely revert to pre-drug treatment state.

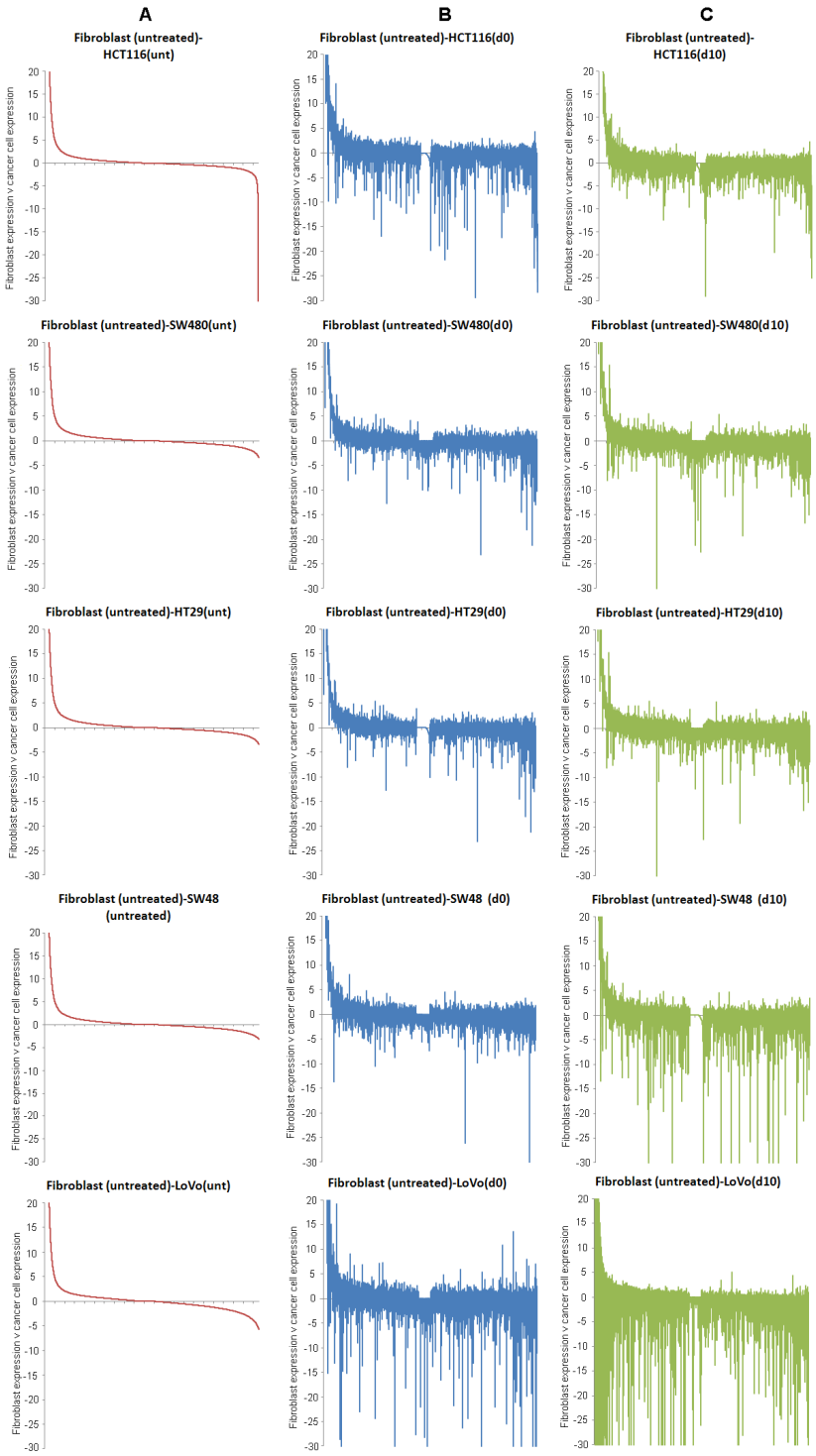

Figure 2 – Changes to gene expression patterns of colorectal cancer cells in comparison to expression patterns of fibroblasts. A panels represent untreated fibroblasts vs untreated cancer cell lines, B panels represent fibroblast vs treated cell lines, C panels represent fibroblast vs 10d post treated cancer cells. Downward peaks represent a gene that is more highly expressed in cancer cells, cells, upward peaks represent a gene a gene that is more highly expressed in normal epithelial cells.

Table 1 – Reactivated genes in colorectal cancer cell lines that constitutively expressed in untreated Fibroblast cells. More than half of the genes in each category are associated with a CpG island.

Time point	CpG Island	HCT116	SW480	HT29	LoVo	SW48
After	Total	595	313	394	865	417
5-aza-dC	CpG+	324	162	201	543	229
treatment	CpG-	271	151	193	322	188
4d post	Total	613	457	427	887	796
5-aza-dC	CpG+	341	253	220	503	474
treatment	CpG-	272	204	207	304	322
10d post	Total	603	459	431	520	484
5-aza-dC	CpG+	307	238	223	293	278
treatment	CpG-	296	221	208	227	216

As predicted in figure 2, 5-aza-dC induced numerous increases in gene expression, and many small yet long-term changes in the cancer cell lines were observed d10 post treatment. To assess the expression status of reactivated genes d10 post treatment, genes were categorised as short term or long term reactivated. With the exception of LoVo cells, more CpG- genes were transiently affected due to 5-aza-dC suggesting that many reactivations were secondary events in response to drug treatment.

Table 2 – Gene expression responses after 5-aza-dC exposure. Many continually expressed genes are associated with a CpG island and more than half of the genes not expressed at any time point do not have a CpG island. Over 1000 previously non-expressed genes were reactivated upon 72h 5-aza-dC treatment. Approximately equal numbers of genes with or without CpG islands were reactivated, 3-fold up or 3-fold down regulated.

Cell Line	CpG Island	Always on	Always off	Reactivated	3X up-reg	3X down-reg
Fibroblast	Total	15120	777	1435	2287	2331
	CpG +	11431	341	707	1143	1166
	CpG -	3689	436	728	1144	1165
HCT116	Total	15938	924	1472	2538	2468
	CpG +	12181	340	739	1321	1296
	CpG -	3757	584	733	1217	1172
HCT116 +TSA	Total	16752	1402	1431	2286	2238
	CpG +	12601	562	672	1106	1074
	CpG -	4151	840	759	1180	1164
SW480	Total	16080	1110	1279	2219	1988
	CpG +	11740	452	638	1168	1342
	CpG -	4340	658	641	1051	646
SW48	Total	15368	691	1551	2751	2648
	CpG +	11754	279	806	1534	1304
	CpG -	3614	412	745	1217	1344
HT29	Total	15867	899	1478	2370	2240
	CpG +	11960	395	782	1290	1090
	CpG -	3907	504	696	1080	1150
LoVo	Total	15137	775	1898	4725	4781
	CpG +	11574	258	1053	3061	2922
	CpG -	3563	517	845	1664	1859

Combined treatment of 5-aza-dC and TSA

Co-treatment of HCT116 cells was performed with 5-aza-dC and TSA in order to allow comparisons and determine synergistic effects on gene reactivation. The HCT116 cell line was chosen as it is a widely studied cell line and the inhibition of histone deacetylase with TSA in these cell lines has been thoroughly described [12]. Combining TSA with 5-aza-dC did not result in greater numbers of gene reactivation in HCT116 cells, however the constant TSA exposure resulted in more genes remaining expressed in the ten day period after 5-aza-dC than without TSA. When co-treated with TSA a synergistic effect on expression was observed in 404 genes. KEGG pathway analysis on these genes identified five significantly affected pathways; primary bile acid biosynthesis, PPAR signalling pathway, tight junction, insulin signalling pathway and synthesis of unsaturated fatty acids. Many of these involve reactivation of silenced tissue specific genes. The AKT protein (part of the tight junction pathway) associated with proliferation became highly over-expressed with co-treatment of 5-aza-dC and TSA. The Akt protein is involved with several pathways including the Apoptosis pathway and may be altered in response to drug treatment in these cells.

Pathway Analysis

The limited efficacy of 5-aza-dC to certain tumour types suggests different pathways of genes are activated, resulting in divergent outcomes. Therefore we sought to identify the pathways affected in colorectal cancer cells following 5-aza-dC treatment.

Genes that experienced a three-fold or greater increase in expression after treatment were analysed to identify pathways altered in three or more cancer cell

up-regulation of related genes as compensation. The Protein Kinase A family is involved in numerous pathways and was found to be significantly altered within the Olfactory transduction pathway of SW48 cells. Although its role in this pathway is likely unrelated to 5-aza-dC treatment, there is a more direct link for the role of PKA within the apoptosis pathway. At least one variant of *PRKACB* gene not expressed in fibroblast cells is down-regulated to non-detectable levels in HT29, SW48 and LoVo cells with 5-aza-dC treatment.

Table 3 – Gene expression patterns 10d post 5-aza-dC treatment. All genes were not repressed in untreated cells. Short term genes reverted to low or non-detectable expression 10d after treatment. Long term genes remained highly expressed 10d after 5-aza-dC treatment. Other refers to any other irregular pattern of expression.

Cell Line	CpG Island	Short Term	Long term	Other
Fibroblasts	Total	626	453	356
	CpG+	272	247	188
	CpG-	354	206	168
HCT116	Total	511	702	244
	CpG+	239	380	111
	CpG-	272	322	133
HCT116 +TSA	Total	354	943	134
	CpG+	166	439	67
	CpG-	188	504	67
SW480	Total	383	650	246
	CpG+	161	357	120
	CpG-	222	293	126
SW48	Total	525	778	248
	CpG+	230	471	105
	CpG-	295	307	143
HT29	Total	551	670	257
	CpG+	272	385	125
	CpG-	279	285	132
LoVo	Total	1116	597	185
	CpG+	644	309	103
	CpG-	472	291	82

Table 4 – Significantly altered pathways containing genes three fold up-regulated in at least three cancer cell lines following 5-aza-dC treatment. Shown are the numbers of genes altered and the percentage of those genes which are within a specific pathway. P-values were generated from the DAVID Functional Annotation tool and represent a modified Fisher Exact P-value.

Pathway	HCT116		SW48		HT29		LoVo		SW480	
	Count (%)	P-value	Count (%)	P-value	Count (%)	P-value	Count (%)	P-value	Count (%)	P-value
Taurine & hypotaurine metabolism	Not significant		Not significant		5 (0.2)	0.031	6 (0.0)	0.0492	Not significant	
Arachidonic acid metabolism	17 (0.2)	0.0015	14 (0.6)	0.0409	17 (0.8)	0.0015	Not significant		Not significant	
Calcium signalling pathway	33 (0.8)	0.0279	40 (1.7)	0.0017	33 (1.5)	0.0279	Not significant		29 (1.5)	0.0325
Cytokine-cytokine receptor interaction	62 (1.5)	$1.8x10^{-6}$	50 (2.1)	0.0167	62 (2.8)	$1.9x10^{-6}$	Not significant		Not significant	
Neuroactive ligand receptor interaction	57 (2.8)	$3.6x10^{-5}$	49 (2.1)	0.017	57 (2.6)	$3.6x10^{-5}$	Not significant		42 (2.2)	0.01
ECM-receptor interaction	18 (2.6)	0.0392	19 (0.8)	0.0385	18 (0.8)	0.0392	Not significant		Not significant	
Hematopoietic cell lineage	24 (0.8)	$4.3x10^{-4}$	22 (0.9)	0.0061	24 (1.1)	$4.27x10^{-4}$	Not significant		17 (0.9)	0.0267
Primary immuno-deficiency	10 (0.5)	0.0308	Not significant		10 (0.5)	0.0308	Not significant		9 (0.5)	0.0355
Olfactory transduction	Not significant		66 (2.8)	0.0395	Not significant		98 (0.1)	0.0409	70 (3.6)	$1.6x10^{-5}$

Table 5 - Significantly altered pathways containing genes three-fold down-regulated in at least three cancer cell lines upon 5-aza-dC treatment. Shown are the numbers of genes altered and the percentage of those genes which are within a specific pathway. P-values were generated from the DAVID Functional Annotation tool. None of these pathways were altered in the SW480 cell line.

Pathway	HCT116		SW48		HT29		LoVo	
	Count (%)	P-value	Count (%)	P-value	Count (%)	P-value	Count (%)	P-value
Cytokine-cytokine receptor	55 (2.6)	0.0003	54 (2.4)	0.0015	Not significant		73 (0.1)	0.0408
Hedgehog signalling pathway	21 (1.0)	$1.1x10^{-5}$	15 (0.7)	0.0154	Not significant		24 (0.4)	0.0025
Allograft rejection	11 (0.5)	0.0136	10 (0.5)	0.0475	Not significant		17 (0.0)	0.0025
Neuroactive ligand receptor interaction	53 (2.5)	0.0005	70 (3.1)	$4.8x10^{-9}$	57 (2.9)	$1.3x10^{-6}$	82 (0.1)	0.0007
Olfactory transduction	86 (4.1)	$8.8x10^{-8}$	92 (4.1)	$9.0x10^{-9}$	100 (5.1)	$3.5x10^{-16}$	112 (0.1)	0.0011
Asthma	10 (0.5)	0.0089	11 (0.5)	0.0036	8 (0.4)	$4.48 x10^{-2}$	17 (0.0)	0.0002

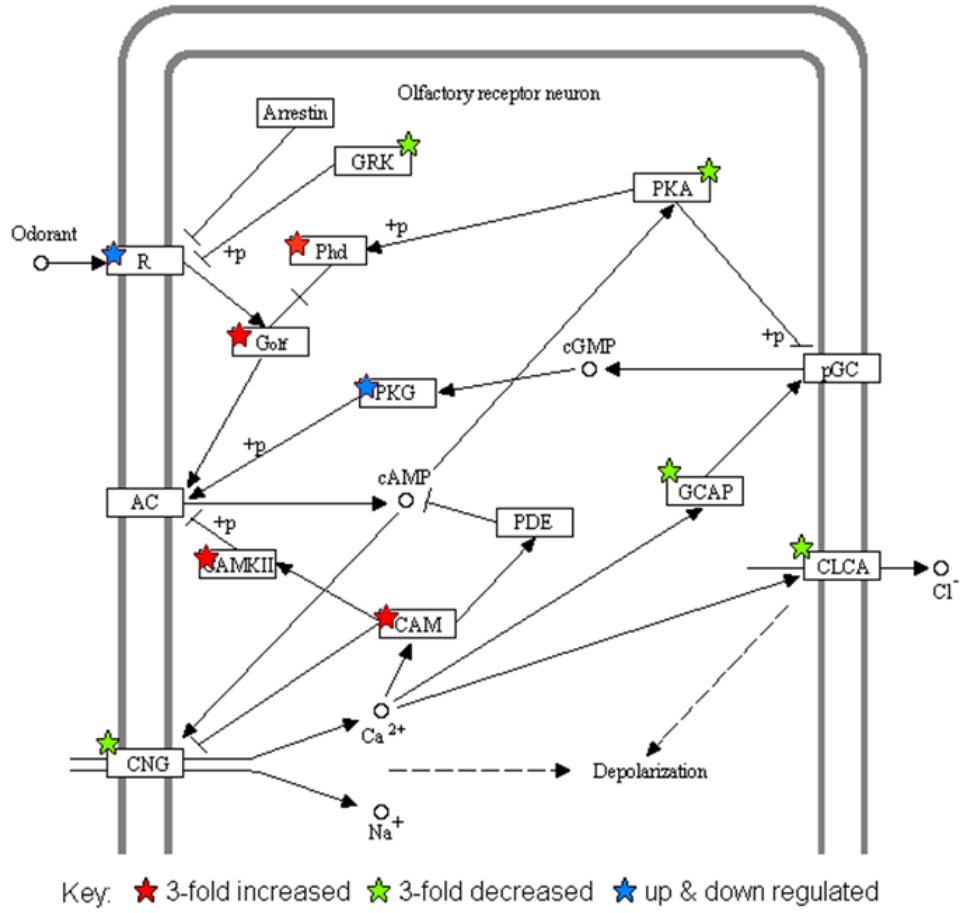

Figure 3 - Affected genes in the Olfactory transduction pathway in SW48 cells. Marked on the KEGG pathway with blue stars are classes of genes affected in both 3-fold up and down regulated with 5-aza-dC treatment. Marked with red stars are genes which were 3-fold up regulated only, green stars are 3-fold down-regulated only and categories with blue stars were affected by both up and down regulated genes.

Discussion

Epigenetic aberrations are a common event in colorectal cancer and include genome wide DNA hypomethylation and site-specific methylation of tumour suppressor genes. Reactivation of silenced genes could prove useful in therapies against such tumours, yet the effectiveness of DNA demethylating agents appears limited to certain tumour types. The success of 5-aza-dC as a treatment of malignancy relies upon its ability to alter patterns of gene expression. When key genes are reactivated, appropriate control mechanisms are re-activated which lead to cell death. As such, we have sought to identify the cellular responses of several colorectal cancer cell lines to treatment with 5-aza-dC and the resulting gene expression profiles.

Genome wide expression changes due to 5-aza-dC exposure

Cellular reactions to 5-aza-dC appear complex and varied between cell types. Exposure to 5-aza-dC does not appear to elicit a certain pattern of gene expression that is common to treated cells on a genome-wide scale. Rather, the genes expressed following treatment appear to be highly dependent on the pre-existing state as several untreated/treated pairs cluster together in an unsupervised analysis. Similarly, d4 and d10 post treatment samples frequently show resemblance, suggesting that the expression pattern changes occur in gradual increments and genome-wide patterns of expression do not necessarily revert to pre-existing levels. Localised changes in DNA methylation can attract further modifications such as histone acetylation, which assist with prolonged reactivation of expression [11]. These patterns may arise from deregulation of the methyltransferase genes. As greater than 95% of methyltransferase activity in

colorectal cancer cells is attributed to the co-operation between DNMT1 and DNMT3B [16], fluctuations in expression of either of these enzymes may dramatically alter the DNA methylation landscape. Additionally, cytotoxicity of 5-aza-dC in mice is mediated by expression of Dnmt3a and Dnmt3b [17], which may account for cell line differences, and also effect resulting gene expression profiles.

Gene expression and CpG islands

A comparison of the gene expression patterns with regard to CpG island status is shown in Table 2. As previously shown, a large number of constitutively expressed genes were associated with a CpG island [18, 19], indicative of DNA hypomethylation - even in the cancer cell lines. Interestingly, approximately half of the genes reactivated with 5-aza-dC are not associated with a CpG island, and a proportion of genes already expressed underwent 3-fold or greater increase in expression. The numbers of this type of expression were consistent across the cell lines assessed. Together, the reactivation of CpG island negative genes and up-regulation of already expressed genes indicates that methylation independent mechanisms [20] such as secondary effects of other reactivated genes are common events in cells exposed to 5-aza-dC.

Alteration of gene expression patterns

Epigenetic modifying agents have previously been used to alter the fate of several particular cell types [10, 21, 22] by inducing changes to gene expression patterns. The expression profiles of untreated fibroblast cells are representative of healthy epithelial cells [23, 24], and comparisons to the treated cancer cell

lines are shown in Figure 2. Gene expression after treatment and 10d post treatment in the cancer cell lines do not resemble that of fibroblasts and suggest that expression levels are still changing 10d after the treatment period. Up to 600 genes reverted from a transcriptionally inactive to active state, equivalent to fibroblasts, but many other genes remained differently expressed and the pattern and level of expression remains complex, particularly in SW48 and LoVo cell lines. Down-regulated genes were abundant, but the magnitude of these changes is more modest than observed in the up-regulated genes. Expression of four transcription factors has recently been associated with a gain of pluripotency [25-27]. Expression of these may be required for a reversion of cancer cells to a normal epithelial cell type and a review of the expression of these genes in our cell line data shows their expression was not co-ordinated which may account for a lack of similarity of expression profiles at any time point.

Generally, with a few exceptions, 5-aza-dC induced more long term reactivation than transient reactivation. Transiently reactivated genes were less often associated with a CpG island, whilst long term reactivated genes were more frequently associated with CpG islands. Reactivation of non-CpG genes with 5-aza-dC signifies a secondary effect, which may render them more susceptible to transient reactivation. As secondary effects are common, 5-aza-dC may indirectly alter gene pathways without epigenetic abnormalities, or pathways not associated with treatment of the targeted disease. Prolonged Trichostatin A treatment in combination with 72h 5-aza-dC exposure caused increased numbers of genes to remain expressed 10d after removal of the demethylating agent. Despite this, the number of reactivated genes previously silenced was not greatly

altered with TSA in the colorectal cancer cells, as previously observed in liver cells [28]. Previous results from our lab have shown that localised hypomethylation at transcription start sites allows long term reactivation [11] and is caused by an increased acetylation of histone H3 (refer to Chapter 5). However, sustained exposure to TSA resulted in increased numbers of long term reactivation of genes when compared with cells treated with 5-aza-dC only, indicating that 5-aza-dC alone may not be sufficient to induce an increased enrichment of H3Ac at genes. The lack of initial genome wide reactivation along with better long term reversion of expression from combined drug treatment suggests a more successful strategy may be to follow up 5-aza-dC treatment with a maintenance course of TSA to extend the period with which reactivated genes can initiate appropriate control mechanisms.

Gene pathway analysis

The identification of specific gene pathways that are affected by 5-aza-dC in colorectal cancer cells may reveal information on the pathways that are associated with effective or non-effective methyltransferase inhibitor treatment. Pathways down-regulated following 5-aza-dC are poorly defined in the literature and may offer an explanation for the lack of effectiveness in solid tumour types. Twenty two genes were observed to become down-regulated as a result of 5-aza-dC treatment, however they appear to be predominantly unrelated in function. Non-specific effects or secondary effects are likely to be responsible for some pathways whilst the Hedgehog Signalling (Wnt proteins) and Cytokine-Cytokine interaction pathways are more relevant to these cells. An insufficient number of transcripts from the apoptosis pathway were affected to render the pathway

significant, but several members of this pathway were commonly affected. A frequent down-regulation of PRKACB variants in the HT29, SW48 and LoVo cell lines was observed whilst these variants are not expressed in the fibroblasts (NM_207578.1 and NM-182948.2). PRKACB is involved with the phosphorylation of BCL2, an agonist of cell death, within the apoptotic pathway, which subsequently leads to cell survival [29, 30]. Similarly BCL2 is an important factor in chemo-resistance [31, 32]. Although *in vitro* evidence suggests apoptosis is induced by 5-aza-dC in colorectal cancer cells [33, 34], it remains ineffective *in vivo*. We detected a pro-apoptotic down-regulation of PRKACB in some cell lines, yet understanding why apoptosis is not actually induced in these tumours *in vivo* poses a challenging task and involves by-passing of this mechanism of cell death.

A previous study in melanoma cells found that six genes implicated in malignant potential were down-regulated by 5-aza-dC [35], but none of these genes correlated with our results indicating this type of response is cell type specific. Data in this study reveals no down-regulated genes related to expected pathways such as cell survival or metastasis that could be used as a general control marker for 5-aza-dC treatment, although the genes in Table 5 are candidates within colorectal cancer cells.

The molecular responses of colorectal cancer cells to 5-aza-dC are complex and appear to be cell line specific. On a genome wide level we did not observe any common pattern of expression, nor did we identify a common biological pathway affected with treatment. Somewhat conflicting is the lack of influence on

apoptotic pathways or cell cycle pathways, but also in concordance with the limited specificity of 5-aza-dC to certain tumours. The down regulation of the *PRKACB* transcripts was commonly observed and these changes are likely to have influenced the phosphorylation of BCL2, which reduces its interaction with apoptosis proteins, yet this mechanism does not appear sufficient to successfully cause cell death in this type of tumour. Interestingly we noted the change in expression of many genes not associated with a CpG island, indicating 5-aza-dC may be able to regulate genes not affected by epigenetic aberrations. The combined reactivation potential of TSA and 5-aza-dC was noted and may be useful for long term reactivation of silenced genes which is required to initiate cell death in particular tumour types.

References

1. Balch C, Yan P, Craft T, Young S, Skalnik D G, et al. (2005) Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. Mol Cancer Ther; 4(10): p. 1505-14.
2. Kantarjian H, Issa J P, Rosenfeld C S, Bennett J M, Albitar M, et al. (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer; 106(8): p. 1794-803.
3. Daskalakis M, Blagitko-Dorfs N, and Hackanson B. (2010) Decitabine. Recent Results Cancer Res; 184: p. 131-57.
4. Yoshida M, Kijima M, Akita M, and Beppu T. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem; 265(28): p. 17174-9.
5. Brunmeir R, Lagger S, Simboeck E, Sawicka A, Egger G, et al. (2010) Epigenetic regulation of a murine retrotransposon by a dual histone modification mark. PLoS Genet; 6(4): p. e1000927.
6. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, et al. (2002) A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet; 31(2): p. 141-9.
7. Zhang Y, Fatima N, and Dufau M L. (2005) Coordinated changes in DNA methylation and histone modifications regulate silencing/derepression of luteinizing hormone receptor gene transcription. Mol Cell Biol; 25(18): p. 7929-39.
8. Chen Z J and Pikaard C S. (1997) Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. Genes Dev; 11(16): p. 2124-36.
9. Pryzbylkowski P, Obajimi O, and Keen J C. (2008) Trichostatin A and 5 Aza-2' deoxycytidine decrease estrogen receptor mRNA stability in ER positive MCF7 cells through modulation of HuR. Breast Cancer Res Treat; 111(1): p. 15-25.
10. Zhang X M, Li Q M, Su D J, Wang N, Shan Z Y, et al. (2010) RA induces the neural-like cells generated from epigenetic modified NIH/3T3 cells. Mol Biol Rep; 37(3): p. 1197-202.
11. Mossman D, Kim K T, and Scott R J. (2010) Demethylation by 5-aza-2'-deoxycytidine in colorectal cancer cells targets genomic DNA whilst promoter CpG island methylation persists. BMC Cancer; 10(1): p. 366.
12. Flatmark K, Nome R V, Folkvord S, Bratland A, Rasmussen H, et al. (2006) Radiosensitization of colorectal carcinoma cell lines by histone deacetylase inhibition. Radiat Oncol; 1: p. 25.
13. Huang da W, Sherman B T, and Lempicki R A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc; 4(1): p. 44-57.
14. Toyota M, Sasaki Y, Satoh A, Ogi K, Kikuchi T, et al. (2003) Epigenetic inactivation of CHFR in human tumors. Proc Natl Acad Sci U S A; 100(13): p. 7818-23.

15. Sato H, Suzuki H, Toyota M, Nojima M, Maruyama R, et al. (2007) Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors. Carcinogenesis; 28(12): p. 2459-66.
16. Rhee I, Bachman K E, Park B H, Jair K W, Yen R W, et al. (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature; 416(6880): p. 552-6.
17. Oka M, Meacham A M, Hamazaki T, Rodic N, Chang L J, et al. (2005) De novo DNA methyltransferases Dnmt3a and Dnmt3b primarily mediate the cytotoxic effect of 5-aza-2'-deoxycytidine. Oncogene; 24(19): p. 3091-9.
18. Saxonov S, Berg P, and Brutlag D L. (2006) A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci U S A; 103(5): p. 1412-7.
19. Medvedeva Y A, Fridman M V, Oparina N J, Malko D B, Ermakova E O, et al. Intergenic, gene terminal, and intragenic CpG islands in the human genome. BMC Genomics; 11(48): p. 48.
20. Schmelz K, Sattler N, Wagner M, Lubbert M, Dorken B, et al. (2005) Induction of gene expression by 5-Aza-2'-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms. Leukemia; 19(1): p. 103-11.
21. Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, et al. (2001) Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation; 68(4-5): p. 235-44.
22. Milhem M, Mahmud N, Lavelle D, Araki H, DeSimone J, et al. (2004) Modification of hematopoietic stem cell fate by 5aza 2'deoxycytidine and trichostatin A. Blood; 103(11): p. 4102-10.
23. Lawrence J B, Oxvig C, Overgaard M T, Sottrup-Jensen L, Gleich G J, et al. (1999) The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A. Proc Natl Acad Sci U S A; 96(6): p. 3149-53.
24. Cronshaw J M and Matunis M J. (2003) The nuclear pore complex protein ALADIN is mislocalized in triple A syndrome. Proc Natl Acad Sci U S A; 100(10): p. 5823-7.
25. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell; 131(5): p. 861-72.
26. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, et al. (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature; 448(7151): p. 318-24.
27. Kunisato A, Wakatsuki M, Kodama Y, Shinba H, Ishida I, et al. Generation of induced pluripotent stem cells by efficient reprogramming of adult bone marrow cells. Stem Cells Dev; 19(2): p. 229-38.
28. Dannenberg L O and Edenberg H J. (2006) Epigenetics of gene expression in human hepatoma cells: expression profiling the response to inhibition of DNA methylation and histone deacetylation. BMC Genomics; 7: p. 181.

29. Chen J, Liu Y, Soh J W, and Aguilera G. (2009) Antiapoptotic effects of vasopressin in the neuronal cell line H32 involve protein kinase Calpha and beta. J Neurochem; 110(4): p. 1310-20.
30. Ohi N, Nishikawa Y, Tokairin T, Yamamoto Y, Doi Y, et al. (2006) Maintenance of Bad phosphorylation prevents apoptosis of rat hepatic sinusoidal endothelial cells in vitro and in vivo. Am J Pathol; 168(4): p. 1097-106.
31. Liao X, Siu M K, Au C W, Wong E S, Chan H Y, et al. (2009) Aberrant activation of hedgehog signaling pathway in ovarian cancers: effect on prognosis, cell invasion and differentiation. Carcinogenesis; 30(1): p. 131-40.
32. Reed J C. (1997) Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin Hematol; 34(4 Suppl 5): p. 9-19.
33. Schneider-Stock R, Diab-Assef M, Rohrbeck A, Foltzer-Jourdainne C, Boltze C, et al. (2005) 5-Aza-cytidine is a potent inhibitor of DNA methyltransferase 3a and induces apoptosis in HCT-116 colon cancer cells via Gadd45- and p53-dependent mechanisms. J Pharmacol Exp Ther; 312(2): p. 525-36.
34. Deng T and Zhang Y. (2009) 5-Aza-2'-deoxycytidine reactivates expression of RUNX3 by deletion of DNA methyltransferases leading to caspase independent apoptosis in colorectal cancer Lovo cells. Biomed Pharmacother; 63(7): p. 492-500.
35. Gollob J A and Sciambi C J. (2007) Decitabine up-regulates S100A2 expression and synergizes with IFN-gamma to kill uveal melanoma cells. Clin Cancer Res; 13(17): p. 5219-25.

Supplementary Table 1 – Previously silenced genes from cancer cell lines that were expressed after 5-aza-dC treatment to match expression as observed in untreated fibroblasts.

Genes in HCT116 whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

Gene	Accession No.	Normalised Exp.	Fibroblasts	Gene	Accession No.	Normalised Exp.	Fibroblasts	Gene	Accession No.	Normalised Exp.	Fibroblasts
ABL2	NM_005158.3	1.42	9.08	CER1	NM_005454.2	1.28	1.89	FLJ44048	NM_207482.2	1.85	0.99
ACP5	NM_001611.2	0.79	1.53	CFL2	NM_021914.5	0.57	5.47	FOXL2	XM_001131060.1	1.24	8.13
ADAM2	NM_001464.3	0.67	1.52	CNGB1	NM_001297.3	1.44	1.08	FPGS	NM_001018078.1	1.80	1.37
AGXT	NM_000030.1	4.31	0.61	CNP	NM_033133.4	0.88	3.78	GGN	NM_152657.3	0.83	1.30
ALOX12	NM_000697.2	4.50	0.78	CNTFR	NM_147164.1	1.56	1.99	GGT6	NM_153338.1	8.30	0.54
ANKRD1	NM_014391.2	1.47	10.85	COL11A1	NM_001854.3	1.08	1.42	GIMAP5	NM_018384.3	1.92	1.30
APBB1IP	NM_019043.3	3.52	92.54	COL6A3	NM_057165.2	1.17	67.90	GLRB	NM_000824.2	5.75	87.42
ARHGEF7	NM_145735.1	2.30	1.28	COX7A1	NM_001864.2	3.04	84.51	GPX7	NM_015696.3	1.40	25.10
ARMCX1	NM_016608.1	4.40	132.40	CRMP1	NM_001014809.1	16.15	1.42	GRIK1	NM_175611.2	0.51	2.01
ARMCX2	NM_177949.1	1.80	35.82	CRYGC	NM_020989.2	0.55	5.16	GRK4	NM_001004056.1	0.98	3.40
ARRDC2	NM_001025604.1	1.42	1.88	CSN2	NM_001891.1	1.62	2.41	GSPT2	NM_018094.2	9.08	31.36
ATP10A	NM_024490.2	1.30	0.87	CST11	NM_080830.2	0.56	1.48	HDGFRP3	NM_016073.2	3.84	14.15
ATP8B2	NM_020452.2	0.66	7.19	CTAG2	NM_020994.2	7.80	1.29	HEATR3	NM_182922.2	0.72	1.44
BASP1	NM_006317.3	2.32	587.79	CYP4A11	NM_000778.2	0.87	0.97	HIST1H2BE	NM_003523.2	1.08	0.89
BCAT1	NM_005504.4	3.89	15.03	DARC	NM_002036.2	0.55	3.02	HIST1H4H	NM_003543.3	1.07	1.20
BHMT2	NM_017614.3	9.97	34.94	DDI1	NM_001001711.2	1.12	1.65	HOM_TES-103	NM_015438.1	0.65	0.77
BRDT	NM_207189.1	13.73	1.81	DGKA	NM_201445.1	0.64	3.26	HOXC10	NM_017409.3	1.06	1.11
C10ORF59	NM_001031709.1	1.53	5.70	DHH	NM_021044.2	1.72	0.62	HRASLS	NM_020386.2	1.10	0.69
C10ORF82	NM_144661.2	2.52	0.69	DHRS9	NM_199204.1	1.63	0.91	HS3ST4	XM_001132212.1	0.83	1.17
C11ORF41	NM_012194.1	2.33	85.94	DNAH11	NM_003777.3	1.40	2.20	HSD17B1	NM_000413.1	3.34	0.81
C15ORF2	NM_018958.2	0.81	1.81	DNALI1	NM_003462.3	1.44	10.11	HSPB6	NM_144617.1	1.46	169.18
C15ORF43	NM_152448.1	3.31	2.96	DOCK10	NM_014689.2	4.25	152.88	HTF9C	NM_022727.4	2.86	1.24
C1ORF51	NM_144697.2	1.47	16.36	DOCK2	NM_004946.1	1.36	26.45	HTR1F	NM_000866.3	1.13	3.73
C2ORF53	NM_178553.3	0.78	1.04	DPPA2	NM_138815.2	9.75	0.59	HTRA1	NM_002775.3	1.87	438.75
C3	NM_000064.2	0.83	1.03	DSEL	NM_032160.2	0.81	124.96	IGFBP7	NM_001553.1	1.24	69.98
C6ORF105	NM_032744.2	0.87	3.88	DUSP15	NM_080611.3	0.89	1.14	IL24	NM_006850.2	2.10	1.85
C6ORF150	NM_138441.2	1.08	1.52	DYSFIP1	NM_001007533.3	2.09	0.56	IL28A	NM_172138.1	0.72	1.43
C8ORF42	NM_175075.3	3.18	9.50	EEA1	NM_003566.3	0.78	3.92	IL6R	NM_000565.2	1.44	0.96
C8ORF79	NM_001039462.1	2.02	0.99	ELA2	NM_001972.2	3.45	3.58	ISLR	NM_005545.3	1.02	172.22
CACNB4	NM_000726.2	2.74	1.08	ELOVL4	NM_022726.2	25.87	35.90	ITGA4	NM_000885.4	0.65	77.53
CART1	NM_006982.1	2.00	1.77	FAM128B	NM_025029.2	1.08	0.62	ITIH5	NM_030569.4	2.36	0.96
CCDC36	NM_178173.2	1.69	0.83	FAM55A	NM_152315.1	0.69	1.28	ITIH5L	NM_198510.1	2.98	2.74
CD3E	NM_000733.2	1.19	1.46	FBN2	NM_001999.3	24.93	15.13	KCNK4	NM_033310.2	1.59	5.78
CD79B	NM_001039933.1	4.58	0.64	FGA	NM_000508.3	1.12	1.31	KCNN2	NM_021614.2	0.70	0.89
CECR1	NM_177405.1	5.79	9.94	FLJ35801	NM_153044.1	1.40	2.29	KIAA0495	NM_207306.2	1.99	20.37

CONT. Genes in HCT116 whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

Gene	Accession No.	Normalised Exp.	Fibroblasts	Gene	Accession No.	Normalised Exp.	Fibroblasts	Gene	Accession No.	Normalised Exp.	Fibroblasts
KIT	NM_001093772.1	0.79	46.85	OTOF	NM_194323.1	2.77	1.14	STON1	NM_006873.2	1.58	18.59
KLC4	NM_201522.1	1.28	0.77	P2RY1	NM_002563.2	1.58	1.77	STXBP1	NM_003165.1	0.98	1.33
KLHL3	NM_017415.1	0.76	7.65	PAQR9	NM_198504.2	1.50	2.03	TAAR8	NM_053278.1	1.17	0.96
KRTAP17-1	NM_031964.1	2.39	0.69	PARVG	NM_022141.4	1.47	0.76	TACR2	NM_001057.1	3.20	1.99
LAYN	NM_178834.3	1.33	309.55	PENK	NM_006211.2	2.20	28.59	TEPP	NM_199456.2	2.34	1.68
LBH	XM_001132517.1	0.55	22.98	PHF21B	NM_138415.2	2.38	0.62	THBS2	NM_003247.2	1.79	107.03
LINGO4	NM_001004432.2	1.98	0.57	PLEC1	NM_201383.1	1.49	0.66	THBS4	NM_003248.3	2.01	1.34
LMO3	NM_001001395.1	1.66	1.44	PNMA3	NM_013364.4	3.52	1.08	THPO	NM_000460.2	3.03	0.85
LOC340156	NM_001012418.2	1.03	1.03	PPAPDC1A	NM_001030059.1	0.75	4.39	THRAP1	NM_005121.2	0.73	0.74
LOC401620	NM_001013688.1	1.02	1.69	PPP1R12B	NM_032103.1	1.69	1.10	THSD1	NM_018676.2	0.98	1.67
LOC51149	NM_016175.3	0.90	0.58	PPYR1	NM_005972.3	1.11	1.77	THY1	NM_006288.2	6.01	842.86
LOC728269	NM_001080790.1	1.56	0.52	PRG-3	NM_017753.2	1.05	1.66	TMEM110	NM_198563.1	1.27	2.10
LOC728343	XM_001127360.1	0.81	1.58	PRNT	NM_177549.2	0.50	0.91	TMEM119	NM_181724.1	0.66	938.44
LOXL4	NM_032211.6	0.93	16.38	PYY2	NM_021093.1	0.80	1.34	TMEM31	NM_182541.2	1.34	0.94
LXN	NM_020169.2	1.45	4.62	RAB14	NM_016322.2	0.98	2.86	TMEM84	NM_173610.1	1.31	3.05
MAGEA11	NM_001011544.1	4.30	0.77	RAB42	NM_152304.1	1.22	1.14	TNFAIP6	NM_007115.2	1.67	8.96
MAGEA12	NM_005367.4	0.91	0.65	RGS20	NM_003702.2	1.47	1.16	TNNT3	NM_001042780.1	3.68	1.45
MAGEC2	NM_016249.2	1.44	0.68	RHBDL2	NM_017821.3	0.54	3.52	TP73L	NM_003722.3	2.54	1.21
MAP3K7IP3	NM_152787.3	1.31	1.00	RLN1	NM_006911.2	1.09	0.64	TRH	NM_013381.1	3.39	3.21
MDM2	NM_006880.2	0.75	4.16	RNF212	NM_194439.1	1.23	3.86	TRHDE	NM_013381.1	4.92	3.21
MEGF11	NM_032445.1	2.00	2.24	SERPINB2	NM_002575.1	0.88	140.75	TRIM22	NM_006074.3	0.95	53.21
MMP21	NM_147191.1	1.69	2.35	SFRP1	NM_003012.3	2.43	34.20	TUBA3E	NM_207312.1	4.26	1.14
MRM1	NM_024864.3	1.02	0.86	SLC15A3	NM_016582.1	0.71	3.23	TUBB2B	NM_178012.3	1.69	0.65
MSRB3	NM_001031679.1	2.97	16.50	SLC25A24	NM_213651.1	0.68	1.86	UCHL1	NM_004181.3	4.83	9.29
NAALAD2	NM_005467.2	1.95	0.89	SLC26A10	NM_133489.2	2.39	1.19	USP26	NM_031907.1	2.38	1.26
NAT2	NM_000015.2	2.74	0.99	SLC2A5	NM_003039.1	4.58	4.58	VAV3	NM_006113.4	1.07	0.75
NDN	NM_002487.2	0.51	181.12	SLC39A5	NM_173596.1	1.76	1.77	VPS13B	NM_015243.2	0.77	1.71
NFASC	NM_001005389.1	0.52	48.87	SLC6A15	NM_018057.4	0.77	1.33	WISP2	NM_003881.2	0.63	16.63
NPFFR2	NM_004885.1	1.63	1.18	SLITRK4	NM_173078.2	1.66	1.08	ZFP3	NM_153018.2	2.99	7.50
NPTX1	NM_002522.2	1.48	13.70	SMARCC2	NM_139067.1	1.02	1.24	ZNF320	NM_207333.2	1.46	2.71
NRCAM	NM_005010.3	2.11	23.87	SOX17	NM_022454.3	2.26	1.16	ZNF334	NM_199441.1	1.35	3.02
OLFM4	NM_006418.3	0.73	1.37	SPAST	NM_014946.3	0.65	1.03	ZNF415	NM_018355.2	1.63	55.35
OLIG3	NM_175747.2	0.75	0.80	SPESP1	NM_145658.2	2.88	11.10	ZNF529	NM_020951.2	1.59	22.83
OLR1	NM_002543.3	0.72	8.02	ST8SIA2	NM_006011.3	1.92	1.58	ZNF606	NM_025027.3	0.88	11.16
OR10AG1	NM_001005491.1	2.60	3.64	STAR	NM_139164.1	0.62	2.47	ZNF662	NM_207404.2	0.99	3.25
OSCAR	NM_130771.3	2.18	1.33	STMN2	NM_007029.2	8.66	269.24	ZPBP2	NM_198844.2	0.70	1.69

Genes in SW48 whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

SW48	Accession No.	Normalised Exp.	Fibroblasts	SW48	Accession No.	Normalised Exp.	Fibroblasts	SW48	Accession No.	Normalised Exp.	Fibroblasts
ADAMTS15	NM_139055.1	1.51	1.46	CECR1	NM_177405.1	0.88	9.94	HCG9	NM_005844.2	1.04	1.45
ADH1C	NM_000669.3	0.63	1.73	CENTB1	NM_014716.2	1.61	4.24	HIST1H1D	NM_005320.2	1.32	0.59
AEBP1	NM_001129.3	0.97	115.98	CER1	NM_005454.2	3.44	1.89	HIST1H2AD	NM_021065.2	1.09	2.29
AK5	NM_174858.1	0.95	57.39	CHODL	NM_024944.2	1.40	0.91	HPX	NM_000613.1	0.62	0.77
ALOX12	NM_000697.2	0.53	0.78	CHRM5	NM_012125.2	0.88	1.89	HSPB6	NM_144617.1	1.38	169.18
AMPD3	NM_001025390.1	0.60	1.29	CMIP	NM_030629.1	1.05	1.54	IL20	NM_018724.3	0.59	0.65
ANKRD42	NM_182603.2	0.51	4.38	COL15A1	NM_001855.3	0.54	38.38	IRAK3	NM_007199.1	1.36	15.89
ARSB	NM_000046.2	1.49	11.13	COL9A2	NM_001852.3	0.59	0.51	ISLR	NM_005545.3	1.35	172.22
ASB4	NM_145872.1	6.93	2.46	CREB3L3	NM_032607.1	0.77	0.63	KCNK12	NM_022055.1	1.00	0.73
BBS12	NM_152618.2	0.58	3.27	CST11	NM_080830.2	1.12	1.48	KIAA1772	NM_024935.2	0.50	14.17
BCHE	NM_000055.2	0.81	3.15	CTF1	NM_001330.2	0.97	17.58	KIF12	NM_138424.1	1.18	0.52
BHMT2	NM_017614.3	1.04	34.94	CTSG	NM_001911.2	1.25	1.71	KIF13A	NM_022113.3	1.00	3.12
BPGM	NM_199186.1	0.99	2.57	DMD	NM_004019.1	1.84	7.51	KLK13	NM_015596.1	2.20	1.81
BRIP1	NM_032043.1	2.32	1.13	DPY19L3	NM_207325.1	1.31	0.87	KRBA1	NM_032534.2	0.93	12.46
BST1	NM_004334.1	1.48	26.25	ECE2	NM_001037324.2	0.82	2.42	LMBRD2	NM_001007527.1	1.65	3.36
C10ORF68	NM_024688.1	0.61	2.29	ELOVL4	NM_022726.2	1.15	35.90	LOC340156	NM_001012418.2	3.45	1.03
C10ORF85	NM_001012711.2	3.32	1.51	EVC2	NM_147127.3	3.84	1.19	LOC389834	NM_001013655.1	0.76	1.19
C13ORF30	NM_182508.1	0.76	1.00	FEZF2	NM_018008.2	1.38	1.81	LOC441426	NM_001013727.1	5.71	0.96
C14ORF178	NM_174943.2	2.85	2.11	FILIP1	NM_015687.2	0.55	1.82	LOC619208	NM_001033564.1	6.54	26.12
C15ORF5	XR_017977.1	1.85	3.41	FLJ43806	NM_201628.1	2.86	1.35	LRRC2	NM_024512.2	1.56	13.60
C1D	NM_006333.2	2.82	2.31	FLJ45537	NM_001001709.1	0.61	1.56	MAML2	NM_032427.1	4.16	5.74
C2ORF52	NM_173513.2	2.18	3.01	FMNL3	NM_198900.2	2.11	0.85	MATN3	NM_002381.4	0.78	2.30
C3ORF15	NM_033364.3	0.50	1.50	FSIP1	NM_152597.4	0.81	3.63	MGC16824	NM_020314.4	2.50	10.52
C3ORF25	NM_207307.1	4.09	0.99	FXN	NM_000144.3	2.18	0.78	MGC34761	NM_173619.2	1.13	1.01
C6ORF128	NM_145316.2	3.01	2.90	GAL3ST3	NM_033036.2	2.93	2.16	MGC42105	NM_153361.2	0.93	22.04
C6ORF25	NM_138276.1	1.36	1.12	GALNTL1	NM_020692.1	2.92	0.56	MGC45800	XR_017780.1	7.05	3.37
C6ORF58	NM_001010905.1	3.27	0.85	GDF3	NM_020634.1	1.47	2.08	NDUFB1	NM_004545.3	1.05	1.94
C9ORF138	NM_153707.1	2.02	0.69	GEFT	NM_182947.2	1.03	6.97	NLRP1	NM_014922.4	1.29	0.85
C9ORF39	NM_017738.2	1.38	4.69	GMFG	NM_004877.1	1.04	3.02	NPM2	NM_182795.1	0.51	0.51
C9ORF43	NM_152786.1	1.53	1.45	GP9	NM_000174.2	2.09	4.08	NTS	NM_006183.3	3.37	1.15
CACNB1	NM_000723.3	0.90	1.89	GPR103	NM_198179.1	1.28	0.59	NXPH1	NM_152745.2	1.08	1.28
CAST	NM_173062.1	0.71	1.27	GPR68	NM_003485.3	2.79	53.14	OR2T1	NM_030904.1	0.81	0.60
CCDC63	NM_152591.1	1.10	0.86	GPX6	NM_182701.1	1.01	1.12	OR2W3	NM_001001957.2	0.88	1.09
CCDC65	NM_033124.3	0.54	1.07	GSPT2	NM_018094.2	0.53	31.36	PADI1	NM_013358.2	2.60	0.69
CCDC96	NM_153376.2	1.53	1.75	HAMP	NM_021175.2	0.71	0.97	PARVG	NM_022141.4	0.83	0.76

CONT. Genes in SW48 whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

SW48	Accession No.	Normalised Exp.	Fibroblasts
PBLD	NM_001033083.1	1.51	1.10
PCDHB12	NM_018932.3	3.84	3.70
PDE8A	NM_173457.1	0.70	0.96
PDIA2	NM_006849.2	0.73	1.60
PIH1D2	NM_001082619.1	2.37	1.02
PNMA3	NM_013364.4	2.43	1.08
PPT2	NM_138717.1	1.11	1.14
PPY	NM_002722.3	0.71	1.35
PRB1	NM_199354.1	0.93	0.75
RASAL2	NM_004841.2	2.01	3.19
RIMS1	NM_014989.3	1.12	7.63
RNF150	NM_020724.1	0.66	70.83
RPH3A	NM_014954.2	1.35	0.55
SCN2A	NM_001040142.1	1.49	0.52
SCNN1B	NM_000336.2	0.72	0.95
SCRN3	NM_024583.2	2.58	4.33
SERPINF1	NM_002615.4	0.57	13.77
SFT2D2	NM_199344.1	1.04	0.98
SKIL	NM_005414.2	0.50	0.89
SLAMF6	NM_052931.3	1.07	1.41
SLC16A6	NM_004694.3	7.67	0.53
SLC30A4	NM_013309.4	1.23	7.11
SLC35E2	NM_182838.1	1.13	2.20
SLC6A1	NM_003042.2	0.79	1.82
SLCO6A1	NM_173488.3	0.62	0.69
SOHLH2	NM_017826.1	0.62	0.75
SPATA9	NM_031952.2	0.75	2.22
SPOCD1	NM_144569.4	0.92	11.17
SYCP2	NM_014258.2	0.87	1.54
TMEM47	NM_031442.2	14.16	14.62
TNNC2	NM_003279.2	1.20	1.33
TNR	NM_018996.2	1.24	0.88
TRH	NM_007117.1	0.80	2.00
TRIM17	NM_016102.2	1.60	2.12
TRIM50	NM_178125.2	2.55	0.74
TRIM55	NM_184085.1	2.97	1.02
TRIM62	NM_018207.1	0.67	1.07
TSPYL5	NM_033512.2	0.68	52.40
TTC30B	NM_152517.2	2.54	3.30
TXLNB	NM_153235.2	1.02	4.51
UMOD	NM_003361.2	1.03	1.26
USP20	NM_001008563.1	0.69	0.66
WDR77	NM_024102.2	0.96	1.50
WFDC10B	NM_172006.2	0.51	1.47
ZFHX2	NM_033400.1	1.25	1.92
ZIC4	NM_032153.3	0.78	152.37
ZNF222	NM_013360.1	0.74	1.22
ZNF454	NM_182594.1	2.11	1.43
ZNF655	NM_001009956.1	2.75	1.21
ZSCAN12	NM_001039643.1	1.37	1.56

Genes in SW480 whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

SW480	Accession No.	Normalised Exp.	Fibroblasts	SW480	Accession No.	Normalised Exp.	Fibroblasts	SW480	Accession No.	Normalised Exp.	Fibroblasts
ACOT7	NM_181865.2	0.70	1.80	FAM127A	NM_001078171.1	0.82	10.51	MEST	NM_002402.2	0.65	1.15
ANGPT2	NM_001147.1	0.98	1.02	FFAR1	NM_005303.1	2.42	2.06	MGC20983	NM_145045.4	1.16	1.82
ANGPTL2	NM_012098.2	2.18	123.42	FILIP1	NM_015687.2	1.67	1.82	MGC33556	NM_001004307.1	3.96	0.58
APBB1IP	NM_019043.3	0.59	92.54	FLJ10781	NM_018215.2	6.79	2.44	MIZF	NM_015517.3	1.11	4.03
AQP12A	NM_198998.1	1.23	0.56	FLJ32065	NM_153032.1	0.57	2.38	MMP23A	NM_004659.1	0.83	3.44
ART4	NM_021071.2	0.68	1.73	FLJ35894	XM_001131199.1	0.99	0.90	MT1M	NM_176870.2	4.78	13.09
BASP1	NM_006317.3	4.70	587.79	FLJ36031	NM_175884.3	2.37	3.02	MYH7	NM_000257.2	0.63	2.21
BCL2L10	NM_020396.2	4.55	0.94	FLJ44186	NM_198508.1	1.05	1.00	MYLC2PL	NM_138403.3	3.22	3.46
BHMT2	NM_017614.3	1.15	34.94	FLJ45202	NM_207507.1	0.93	1.13	MYO1G	NM_033054.1	0.81	0.83
C10ORF72	NM_001031746.2	0.78	25.09	FPRL1	NM_001005738.1	1.38	3.58	NAALADL1	NM_005468.2	2.19	28.75
C15ORF43	NM_152448.1	0.86	2.96	GABBR2	NM_005458.5	1.38	680.99	NEURL	NM_004210.3	0.98	0.57
C18ORF2	NM_031416.1	0.55	2.43	GABRA4	NM_000809.2	0.85	3.28	NOX5	NM_024505.2	1.47	3.87
C1ORF100	NM_001012970.1	2.68	1.29	GALC	NM_000153.2	2.18	114.50	OR2T11	NM_001001964.1	0.71	0.52
C1ORF2	NM_006589.2	1.44	1.44	GCK	NM_033508.1	9.08	2.06	OR2T33	NM_001004695.1	0.97	0.63
C1ORF51	NM_144697.2	2.82	16.36	GCM2	NM_004752.2	0.55	2.31	OR52I2	NM_001005170.1	2.14	1.39
C6ORF78	NM_153036.1	0.62	1.21	GGTL3	NM_178025.1	1.00	1.58	OR5M1	NM_001004740.1	0.82	2.87
C6ORF79	NM_022102.1	0.70	2.56	GML	NM_002066.1	1.04	0.61	OXT	NM_000915.2	1.03	0.98
CAMP	NM_004345.3	1.15	0.83	GPR103	NM_198179.1	1.20	0.59	PHACTR1	NM_030948.1	1.08	12.91
CD7	NM_001039933.1	1.68	0.64	GSPT2	NM_018094.2	0.52	31.36	PIAS2	NM_173206.2	0.93	2.10
CLEC1A	NM_016511.2	0.51	1.11	HIST1H2AD	NM_021065.2	2.19	2.29	POMC	NM_001035256.1	25.22	2.95
COL9A2	NM_001852.3	0.93	0.51	HIST1H3E	NM_003532.2	1.31	0.74	PPAPDC3	NM_032728.2	0.67	42.59
COMP	NM_000095.2	1.08	326.59	HR	NM_005144.3	0.99	1.52	PPP1R3A	NM_002711.2	0.59	1.43
CORIN	NM_006587.2	0.93	1.27	ICOS	NM_012092.2	0.64	1.36	PTGER1	NM_000955.2	3.08	1.58
CRYAA	NM_000394.2	1.03	0.53	IGSF2	NM_004258.2	1.06	2.16	RAB34	NM_031934.3	3.60	27.62
CTAG1A	NM_139250.1	2.34	0.73	KLK15	NM_138564.1	14.59	0.93	RASGRP2	NM_153819.1	0.76	0.50
CTSF	NM_003793.3	1.58	19.10	KRBA1	NM_032534.2	2.36	12.46	RGMB	NM_173670.2	1.04	13.53
CUGBP2	NM_001025077.2	0.89	0.86	KREMEN1	NM_032045.3	0.93	0.95	RNASE4	NM_194431.1	0.85	3.30
CYP27C1	NM_001001665.2	1.82	5.53	L1TD1	NM_019079.2	5.07	1.93	RNF186	NM_019062.1	0.63	0.54
DENND2A	NM_015689.2	1.29	2.45	LCTL	NM_207338.2	1.50	3.65	SBSN	NM_198538.1	1.00	0.91
DIP2A	NM_206889.1	0.84	1.04	LDLRAD2	NM_001013693.1	0.94	0.92	SCNN1G	NM_001039.3	1.15	0.77
DNALI1	NM_003462.3	1.07	10.11	LHFPL4	NM_198560.2	0.97	0.93	SERPINB12	NM_080474.1	0.63	1.49
DPF3	NM_012074.2	2.46	2.20	LOC388284	NM_001012984.2	1.29	13.18	SLC15A3	NM_016582.1	0.69	3.23
EBI2	NM_004951.3	1.34	2.36	LOC441476	NM_001004353.2	2.58	1.40	SLC28A2	NM_004212.2	1.52	1.26
EMILIN1	NM_007046.1	1.94	98.38	MAGEH1	NM_014061.3	1.09	13.45	SLC2A5	NM_003039.1	4.57	4.58
ENG	NM_000118.1	1.06	20.63	MASP1	NM_001031849.1	1.99	13.04	SP140	NM_007237.3	0.66	1.03

CONT. Genes in SW480 whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

SW480	Accession No.	Normalised Exp.	Fibroblasts
SPON1	NM_006108.2	3.15	1.11
ST8SIA2	NM_006011.3	1.62	1.58
TAF7L	NM_024885.2	11.45	1.15
TARP	NM_001003806.1	0.74	0.53
TCP11	NM_001093728.1	2.01	1.66
TEPP	NM_199456.2	2.87	1.68
THAP8	NM_152658.2	2.53	4.33
THY1	NM_006288.2	62.25	842.86
TIAM2	NM_012454.3	1.25	1.33
TNFAIP8L3	NM_207381.2	0.51	1.33
TNNC2	NM_003279.2	1.00	1.33
TRIM34	NM_130390.1	1.23	0.91
TRIM50	NM_178125.2	14.10	0.74
TRIM55	NM_184085.1	3.04	1.02
TSHZ2	NM_173485.4	1.39	65.91
TSKS	NM_021733.1	7.94	3.04
TUB	NM_006001.1	48.33	75.14
UGCGL1	NM_001025777.1	1.61	1.37
ZNF256	NM_005773.2	4.89	21.84
ZNF285A	NM_152354.3	0.59	15.26
ZNF667	NM_022103.2	0.68	14.43
ZNF772	NM_001024596.1	3.12	10.04
ZNF776	NM_173632.2	0.82	1.23
ZNF781	NM_152605.2	4.65	2.50
ZSCAN18	NM_023926.3	1.17	161.97

Genes in LoVo whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

LoVo	Accession No.	Normalised Exp.	Fibroblasts	LoVo	Accession No.	Normalised Exp.	Fibroblasts	LoVo	Accession No.	Normalised Exp.	Fibroblasts
ABCA1	NM_005502.2	0.74	2.39	DSEL	NM_032160.2	0.83	124.96	LOC728946	XM_001128870.1	0.91	82.69
ABCA13	NM_152701.2	3.03	9.32	DYNLRB2	NM_130897.1	0.68	11.39	LRRC43	NM_152759.4	3.90	2.78
ABCA6	NM_080284.2	2.05	5.34	EPHB1	NM_004441.3	1.05	27.39	LUM	NM_002345.3	27.21	2409.92
ADAM12	NM_021641.2	1.07	9.09	EPYC	NM_004950.3	2.16	1.87	LYPD2	NM_205545.1	0.81	0.73
ADAMTSL1	NM_139238.1	1.73	3.16	FBLN1	NM_006487.2	1.00	6.19	MFSD7	XM_001127310.1	1.04	6.76
AEBP1	NM_001129.3	0.70	115.98	FGF2	NM_002006.4	25.09	51.57	MT1E	NM_175617.2	1.05	1.08
AMOTL1	NM_130847.1	1.29	2.43	FLJ37396	NM_173671.1	1.00	0.52	MYH7	NM_000257.2	1.09	2.21
ANGPT1	NM_001146.3	5.11	102.32	FLRT1	NM_013280.4	0.69	2.37	NAALADL1	NM_005468.2	1.10	28.75
ANPEP	NM_001150.1	1.85	75.41	FOXL2	NM_023067.2	1.30	12.23	NFASC	NM_001005388.1	2.17	4.22
AP3B2	NM_004644.3	1.55	1.75	GABRB3	NM_000814.4	1..75	6.69	NPTX1	NM_002522.2	19.31	13.70
ATP6V1C1	NM_001695.4	2.61	1.20	GALC	NM_000153.2	2.01	114.50	NR3C1	NM_001020825.1	0.99	14.54
AVIL	NM_006576.2	1.95	1.86	GPR68	NM_003485.3	1.44	53.14	OR10AG1	NM_001005491.1	5.30	3.64
BHMT2	NM_017614.3	4.67	34.94	GPX7	NM_015696.3	1.80	25.10	OR1Q1	NM_012364.1	1.95	0.77
C11ORF70	NM_032930.1	1.29	19.04	GRIA3	NM_000828.3	3.28	39.04	OR9G9	NM_001013358.1	2.89	1.26
C13ORF21	NM_001010897.1	0.95	33.48	HECW2	NM_020760.1	1.19	33.11	PDZK1	XM_001126710.1	0.76	2.32
C16ORF73	NM_152764.1	1.43	0.79	HHIP	NM_022475.1	0.59	1.43	PDZRN3	XM_001133042.1	1.25	35.79
C20ORF102	NM_080607.2	0.68	3.39	HOXD10	NM_002148.3	5.01	17.33	PKD2	NM_000297.2	0.78	18.11
C20ORF179	NM_001014977.2	1.94	0.85	HSD17B1	NM_000413.1	0.78	0.81	PRDM5	NM_018699.2	1.48	3.47
C21ORF42	NM_058184.1	0.98	1.40	HTR1F	NM_000866.3	1.59	3.73	PSCA	NM_005672.3	0.83	0.51
C2ORF53	NM_178553.3	0.61	1.04	IGFALS	NM_004970.1	0.89	1.44	PSG3	NM_021016.3	1.64	195.06
C8ORF47	NM_173549.1	0.73	1.14	IGSF10	NM_178822.3	1.95	0.67	PTPRC	NM_080923.2	2.92	1.75
C9ORF121	NM_145283.1	0.95	6.52	IL13RA2	NM_000640.2	1.59	23.09	PXDNL	NM_144651.2	1.67	2.65
CCDC67	NM_181645.3	0.87	0.79	INA	NM_032727.2	10.77	3.83	RAB34	NM_031934.3	1.19	27.62
CDH11	NM_014522.1	1.24	560.72	IRAK3	NM_007199.1	0.57	15.89	RBPMS	NM_006867.2	1.01	18.56
CDH2	NM_001792.2	1.61	491.96	ITGB8	NM_002214.2	9.02	2.11	RLBP1L1	NM_173519.1	1.69	1.07
CECR1	NM_177405.1	2.06	9.94	KCNK16	NM_032115.2	0.86	1.90	RNF150	NM_020724.1	1.84	70.08
COPZ2	NM_016429.2	0.70	141.76	KIAA1199	NM_018689.1	0.74	17.73	RNF212	NM_194439.1	0.81	3.86
COX6A2	NM_005205.2	2.73	3.06	KIT	NM_001093772.1	1.27	46.85	RTN4	NM_007008.2	2.42	2.15
CTGF	NM_001901.1	1.02	22.73	KRT33A	NM_004138.2	1.83	1.94	S100A12	NM_005621.1	2.71	1.45
CTHRC1	NM_138455.2	1.00	12.70	LOC196549	NM_145293.2	0.95	5.05	S100A7	NM_002963.3	0.62	2.22
CYLD	NM_001042412.1	0.65	6.98	LOC388965	NM_001013648.3	4.47	0.88	SHBG	NM_001040.2	2.86	3.63
CYSLTR2	NM_020377.2	1.72	1.87	LOC441426	NM_001013727.1	1.16	0.96	SHC4	NM_203349.2	1.74	4.03
DRP2	NM_001939.2	2.82	1.57								

CONT. Genes in LoVo whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

LoVo	Accession No.	Normalised Exp.	Fibroblasts
SLC28A2	NM_004212.2	6.34	1.26
SPESP1	NM_145658.2	1.70	11.10
SPRYD5	NM_032681.1	306.00	1.58
STOM	NM_004099.4	0.98	4.27
SULT1C3	NM_001008743.1	1.89	1.48
TBX15	NM_152380.2	0.75	58.48
TCEAL2	NM_080390.3	0.84	1.15
TCEAL7	NM_152278.2	1.89	42.45
TCP11	NM_001093728.1	1.04	1.66
TMEM31	NM_182541.2	0.94	0.94
TRIM55	NM_184085.1	0.69	1.02
TSPYL2	NM_022117.1	0.62	1.04
TUSC1	NM_001004125.2	1.48	31.53
TYROBP	NM_003332.2	0.75	1.09
USH2A	NM_206933.1	0.53	2.06
VIPR2	NM_003382.3	1.54	1.39
WDFY3	NM_178585.1	0.95	0.60
ZFP3	NM_153018.2	2.65	7.50
ZIC4	NM_032153.3	1.20	152.37
ZIK1	NM_001010879.2	0.79	5.63
ZNF233	NM_181756.1	0.91	4.63
ZNF493	NM_175910.4	1.18	3.79
ZNF626	NM_145297.3	1.28	25.19
ZSCAN18	NM_023926.3	1.59	161.97

Genes in HT29 whose expression is equivalent to fibroblast expression after 5-aza-dC treatment

HT29	Accession No.	Normalised Exp.	Fibroblasts	HT29	Accession No.	Normalised Exp.	Fibroblasts	HT29	Accession No.	Normalised Exp.	Fibroblasts
ABCA5	NM_018672.2	1.11	5.25	EN2	NM_001427.3	0.99	0.60	PCTK2	NM_002595.2	0.51	1.54
ACVR1B	NM_020327.2	1.38	1.46	EYA4	NM_172105.2	1.54	0.69	PDZRN3	XM_001133042.1	4.06	35.79
AGL	NM_000028.2	0.52	0.95	G3BP2	NM_012297.3	1.43	1.83	PLEKHB2	NM_001031706.1	1.07	2.29
AGTR1	NM_000685.4	1.50	13.59	GARNL4	NM_015085.3	0.97	0.93	POMC	NM_001035256.1	1.14	2.95
AIF1	NM_032955.1	1.26	0.65	GOLGA8B	NM_001023567.1	3.30	0.85	POPDC2	NM_022135.2	0.70	4.26
AKAP4	NM_139289.1	1.66	1.67	GTPBP1	NM_004286.4	0.93	1.02	PPP2R2B	NM_181676.1	3.79	5.38
ANGPT2	NM_001147.1	1.40	1.02	GTPBP3	NM_133644.1	1.37	1.03	RARA	NM_000964.2	0.90	0.84
ANGPTL4	NM_139314.1	1.01	2.98	IFIT3	NM_001031683.1	1.06	4.07	RDH8	NM_015725.2	0.92	0.57
AOX1	NM_001159.3	1.04	51.48	IGSF2	NM_004258.2	1.36	2.16	REG3A	NM_138938.1	1.00	1.84
ARMC4	NM_018076.2	0.84	8.30	IQUB	NM_178827.3	2.89	0.92	RFPL3	NM_001098535.1	2.90	1.11
ASCL1	NM_004316.2	3.25	2.77	KCNG1	NM_172318.2	0.67	1.20	RHOJ	NM_020663.3	2.03	63.09
ASL	NM_006477.3	1.10	1.31	KIAA1822L	NM_024746.3	0.53	1.77	ROBO1	NM_133631.1	0.99	32.96
AURKC	NM_001015879.1	3.21	0.50	KIF25	NM_005355.3	0.84	1.14	RTN4	NM_007008.2	0.80	2.15
BCL2L14	NM_030766.1	1.24	1.76	KLF8	NM_007250.3	0.98	1.90	SERHL	NM_170694.1	1.38	3.43
BPIL1	NM_025227.1	1.54	1.51	KREMEN1	NM_032045.3	0.92	0.95	SERPINF1	NM_002615.4	0.59	13.77
BTN3A1	NM_007048.4	0.55	1.00	LBP	NM_004139.2	1.75	2.12	SGCG	NM_000231.1	1.11	9.95
C13ORF16	NM_152324.1	1.19	0.85	LOC147650	NM_207324.1	0.93	0.81	SOX5	NM_152989.2	1.21	1.64
C17ORF42	NM_024683.2	1.16	2.10	LRCH2	NM_020871.3	0.67	14.43	SOX6	NM_017508.1	1.99	1.07
C20ORF75	NM_152611.2	1.72	0.98	LRRC10	NM_201550.2	1.38	1.22	SV2C	NM_014979.1	3.07	0.68
C8ORF31	NM_173687.2	1.07	5.53	LRSAM1	NM_138361.3	1.62	0.97	TIGD7	NM_033208.2	1.85	2.22
CACNA1A	NM_000068.3	1.12	5.12	MAGEH1	NM_014061.3	1.00	13.45	TMSL8	NM_021992.2	3.04	0.61
CD300E	NM_181449.1	0.93	2.00	MORN3	NM_173855.3	1.74	2.24	TNNT3	NM_001042780.1	1.40	1.45
CDC14B	NM_033332.1	0.53	2.55	MXRA8	NM_032348.2	0.96	74.10	TRAM1L1	NM_152402.2	1.49	5.16
CG018	NM_001079691.1	0.53	3.76	MYO1G	NM_033054.1	0.80	0.83	TRERF1	NM_033502.1	0.52	1.06
CLSTN3	NM_014718.3	1.38	0.54	NLGN3	NM_018977.2	0.96	0.74	VEGFA	NM_001025366.1	1.89	0.76
COL4A1	NM_001845.4	1.14	107.06	NPAS4	NM_178864.2	2.46	0.52	ZFP28	NM_020828.1	0.62	6.89
COL9A2	NM_001852.3	0.50	0.51	NR2E1	NM_003269.2	0.67	1.13	ZIK1	NM_001010879.2	1.48	5.63
CTRB2	NM_001025200.3	0.85	1.86	NR3C1	NM_001018077.1	2.73	1.13	ZNF439	NM_152262.2	1.02	21.96
CXORF55	XR_017698.1	0.63	0.58	NXPH3	NM_007225.1	1.85	3.02	ZNF521	NM_015461.1	2.65	306.16
DBX2	NM_001004329.1	1.06	0.80	OPN1SW	NM_001708.1	1.30	2.43	ZNF541	NM_032255.1	1.09	0.83
DGKZ	NM_201532.1	2.29	1.70	OR52E8	NM_001005168.1	1.00	0.54	ZNF571	NM_016536.2	2.19	0.79
DIP2A	NM_206891.1	0.97	0.82	OR6C70	NM_001005499.1	0.77	0.64	ZNF750	NM_024702.2	1.85	1.29
DNALI1	NM_003462.3	0.95	10.11	OR9G9	NM_001013358.1	1.91	1.26	ZPBP	NM_198844.2	0.55	1.69
DRD4	NM_000797.2	1.18	0.72	PCDHGA10	NM_018913.2	1.80	2.02	ZXDA	NM_007156.3	2.73	2.08

Chapter 5

Long term transcriptional reactivation of epigenetically silenced genes in colorectal cancer cells requires both DNA hypomethylation and histone acetylation

STATEMENT IV

This statement explains the contribution of all authors in the article listed below:

Mossman, D. and Scott, R.J., (2009) Long term transcriptional reactivation of epigenetically silenced genes in colorectal cancer requires both DNA hypomethylation and histone acetylation. Submitted to The International Journal of Cancer, October 2010.

Table IV: Author contribution Percentage and Description of Contribution to the article listed above.

Author	Contribution (%)	Description of Contribution to Article	Signature
David Mossman	90%	Executed the study. Analysed and interpreted the data. Wrote the manuscript.	
Rodney J. Scott	10%	Designed the study, provided the concept and corrected the manuscript.	

Chapter Introduction:

This study was a follow up to the work presented in Chapter 3 in order to characterise the histone protein modifications that are associated with the expression level of a reactivated gene. Our previous study found that after 5-aza-dC exposure there was very little change to DNA methylation of specific genes despite a dramatic increase in expression, and that the DNA methylation pattern could not adequately explain gene expression levels following drug treatment. Here we analysed changes in histone protein modifications that combine with DNA methylation patterns to determine expression levels after 5-aza-dC exposure. The results indicated that genes with localised hypomethylation at the transcription start site were expressed for ten or more days after 5-aza-dC exposure. Elevated expression was due in part to a decrease of the repressive histone 3 lysine 9 and 27 trimethylation protein modifications, but also an increase in the transcriptional-permissive acetylation of histone H3. The reactivation of genes in this study demonstrate how DNA methyltransferase and histone deacetylase inhibitors may have synergistic effects in the reactivation of epigenetically silenced genes, which assist with the development of targeted treatments of malignancy.

Long term transcriptional reactivation of epigenetically silenced genes in colorectal cancer cells requires DNA hypomethylation & histone acetylation

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Running title: Hypomethylation, acetylation and long term gene reactivation

Key Words: methylation, acetylation, 5-aza-2'-deoxycytidine, remethylation, colorectal cancer

Abstract

Epigenetic regulation of genes involves the coordination of DNA methylation and histone modifications to maintain transcriptional status. These two features are frequently disrupted in malignancy such that critical genes succumb to inactivation. 5-aza-2'-deoxycytidine (5-aza-dC) is an agent which inhibits DNA methyltransferase, and holds great potential as a treatment for cancer, yet the extent of its effectiveness varies greatly between tumour types. We aimed to identify epigenetic changes involved with short and long term gene reactivation following 5-aza-dC exposure. Two colorectal cancer cell lines, HCT116 and SW480, were treated with 5-aza-dC and then grown in drug-free media to allow re-methylation. Bisulfite sequencing and Chromatin Immuno-Precipitation analysis were performed on reactivated genes to characterise the epigenetic changes associated with transcription. Increased H3 acetylation, H3K4 tri-methylation and loss of H3K27 tri-methylation were associated with reactivation. Three reactivated genes studied, *CDO1*, *HSPC105* and *MAGEA3*, which were still expressed 10 days post 5-aza-dC treatment displayed localised hypomethylation at the transcriptional start site and also an increased enrichment of histone H3 acetylation. Hypermethylated genes that did not show increased acetylation were transiently expressed with 5-aza-dC treatment before reverting to an inactive state. These observations suggest that Histone H3 acetylation and localised hypomethylation must occur for long term reversion of these epigenetically silenced genes and demonstrate how DNA methyltransferase and histone deacetylase inhibitors cooperate to reactivate silenced genes.

Introduction

The human genome contains several million base pairs of DNA that require strategic packaging into a compact, yet dynamic structure. Condensation is achieved with the supercoiling of ~147 bp DNA around an octamer of histone proteins (two copies of each H2A, H2B, H3 and H4) to form a nucleosome [1] which impedes accidental gene expression and increases the dependence of transcriptional activators [2]. Transcriptional control is primarily mediated by DNA methylation and is assisted by extensive modifications at highly conserved lysine residues on the tails of histone proteins. Lysine acetylation facilitates transcription by weakening the association of the histone and DNA [3] and allows transcription factor binding [4]. Lysine methylation is more complex and can be associated with both active and repressed regions of DNA, and may be present in mono-, bi-, and tri-methylated forms [5]. For instance, trimethylation of histone H3 lysine 4 (H3K4me3) is an active mark [6] whilst methylation of H3K9 and H3K27 appears at transcriptionally silent gene promoters [6, 7].

Aberrant epigenetic silencing of genes can initiate malignancy and frequently appears in addition to genetic alterations, contributing to disease progression in several forms of cancer [8-10]. Reduced expression of numerous genes due to epigenetic silencing correlates with poor prognosis in many forms of malignancy such as lung [11], melanoma [12], breast [13], gastric [14] and colon [15]. Rare instances of soma-wide mono-allelic methylation of *MLH1* have been shown to arise via germline transmission [16]. In addition, heritable copy-number variations can result in transcriptional read through and in-*cis* methylation when adjacent to key genes [17]. These mechanisms offer an explanation of why some families are at a higher risk of disease development despite not carrying an underlying genetic mutation of crucial

genes. Individuals within such families could benefit from early detection of aberrant epigenetic marks at genes which confer an elevated risk of a particular disease. With an increasing awareness of epigenetic abnormalities in disease, counteracting these changes with methyltransferase inhibitors such as 5-aza-2'-deoxycytidine (5-aza-dC) would appear to be a potentially effective treatment. In reality, this treatment effective in a specific group of tumour types [18], which may be due to reactivated genes reverting to a silenced state upon cessation of treatment.

We have previously identified the reactivation of numerous genes in colorectal cancer (CRC) cell lines following treatment with the demethylating agent 5-aza-dC [19]. Upon removal of the drug and ten days of growth, some of these genes remained highly expressed, suggesting a reversal of the transcriptional status of these genes. Although reduced by 5-aza-dC, the changes in DNA methylation did not correlate with the levels of expression in the group of genes analysed, indicating other epigenetic modifications were controlling transcription. In this study we characterised the changes of DNA methylation and chromatin state which allow for either a long or short term reactivation of expression following 5-aza-dC exposure.

Results

Genomic DNA Methylation with 5-aza-dC treatment

Global methylation levels decreased following 5-aza-dC treatment by 53% and 59% in the HCT116 and SW480 cell lines respectively (figure 1). This represents a significant decrease compared with cells that were mock treated which did not undergo demethylation (HCT116 p-value=0.003, SW480 p-value=0.017). Continued incubation of cells for a further ten days after treatment in drug free media allowed re-methylation and genomic levels increased, but did not return to pre-treatment levels in this period.

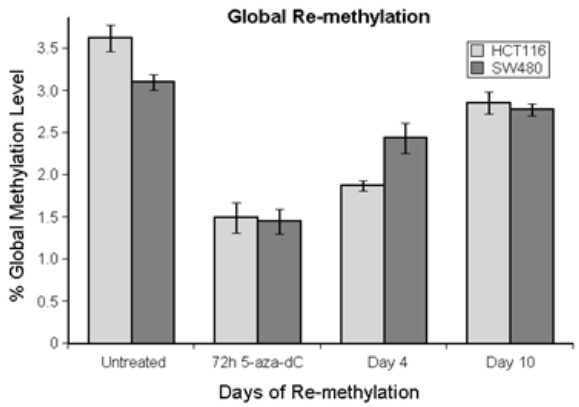


Figure 1 - Genome wide methylation levels and 5-aza-dC treatment. Genomic methylation levels decreased significantly in both cell lines following 5-aza-dC exposure. Levels were gradually restored over the next ten days of drug-free growth and were approaching pre-treatment levels.

Gene Specific methylation and re-expression with 5-aza-dC treatment

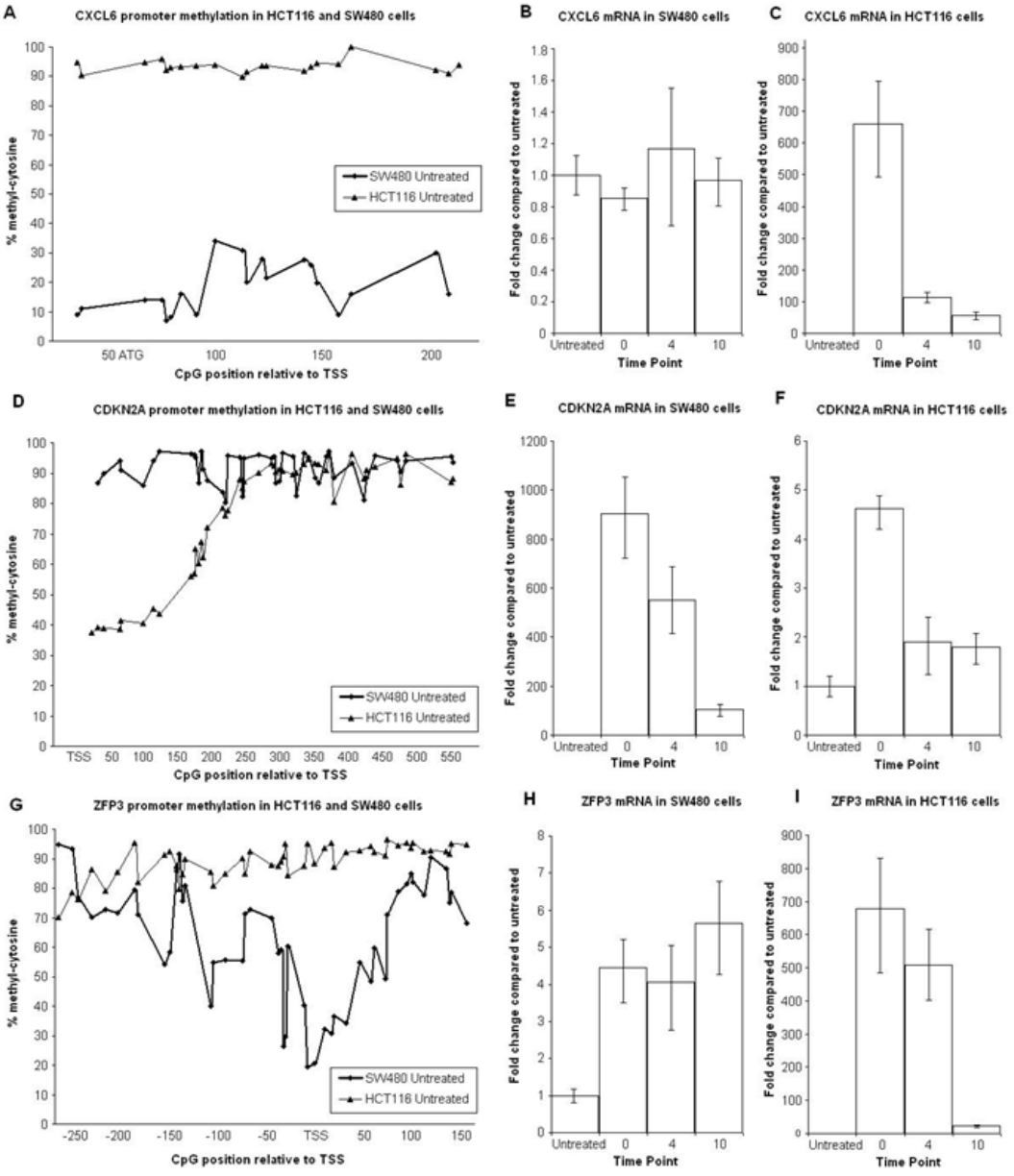
Using genome wide expression arrays we have previously identified patterns of transient and long term gene reactivation following 5-aza-dC treatment [19]. The same genes were again examined in this study to allow characterisation of histone modifications. In this experiment, expression was determined with quantitative PCR and genes were then classified into four categories; always-expressed, up-regulated,

short term reactivated or long term reactivated, as listed in Table 1. Genes were previously selected to allow comparison of short or long term expression against genes with an 'always-expressed' or 'up-regulated' expression pattern. Criteria for long term reactivated genes were as follows; not expressed in untreated cells, detectable expression following treatment and four days post-treatment, and day 10 post treatment expression >100-fold higher than untreated (based on a C_T value of 40 for undetectable transcripts). Criteria for short term expressed genes was similar, with the exception of day ten expression, which was required to be <100-fold above the level of untreated cells.

The three genes which were reactivated for a short period only (*CXCL6* and *ZFP3* in HCT116 cells and *CDKN2A* in SW480 cells) were all hypermethylated across the assayed region of their CpG islands. The expression pattern of these genes was markedly different in the other of the two cell lines; here, these genes showed very little or low methylation at the transcription start site (TSS) and were either expressed continually or became up-regulated (figure 2). The genes which remained highly expressed after reactivation (*CDO1*, *HSPC105*, *MAGEA3*) displayed unique methylation profiles and featured a hypomethylated CpG site adjacent to the transcription start site (TSS) as shown in figure 3. The CpG island specific demethylation after treatment was minimal compared to the genomic demethylation as previously observed [19], therefore only untreated methylation patterns are shown in Figure 2 and 3.

Table 1: Generalised epigenetic events that occur in the four main groups of gene expression.

	Continuously Expressed Genes	Up-regulated Genes	Short Term Reactivated genes	Long Term Reactivated genes
Genes:	*CXCL6* (SW480), *CDKN2A*, *HSPC105*, *MAGEA3* (HCT116)	*ZFP3* (SW480), *CDO1* (HCT116)	*CDKN2A* (SW480), *CXCL6*, *ZFP3* (HCT116)	*CDO1*, *HSPC105*, *MAGEA3* (SW480)
Untreated cells	Expressed. Widespread hypomethylation or hypomethylation near the TSS. H3Ac and H3K4me3 are predominant modifications.	Lowly expressed in untreated cells. Varied levels of DNA methylation. Varied levels active/repressive modifications.	Not expressed. Uniform hypermethylation. H3K4me3 and H3K9me3 predominant.	Not expressed. Unique hypomethylation at TSS. Varied amounts of active / repressive modifications.
Treated cells (72 h)	Stable or increased expression. Minimal demethylation. Stable or slightly increased H3Ac & H3K4me3.	Increased expression. Minimal demethylation. Increased H3Ac, H3K4me3 and H3K9me3.	Expression detected. Minimal demethylation. Stable or non-significant increase in H3Ac. Increased H3K4me3. Increased or stable H3K9me3.	Expression detected. Minimal demethylation. Increased H3Ac, H3K4me3 and H3K9me3.
Ten days post 5-aza-dC treatment	Stable expression. Promoter methylation at pre-treatment level. Increased or stable H3Ac and H3K4me3. Decreased H3K H3K27me when present.	Elevated and / or receding expression. Promoter methylation at pre-treatment level and pattern. Increased H3K4me3.	Expression down-regulated or approaching pre-treatment levels. Promoter methylation at pre-treatment levels. H3K4me3 stable or remains elevated.	Expression remains high. Promoter methylation at pre-treatment level. H3K9me3 stable. Increased H3K4me3 / H3Ac. H3K27me3 reduced (if high in untreated cells).



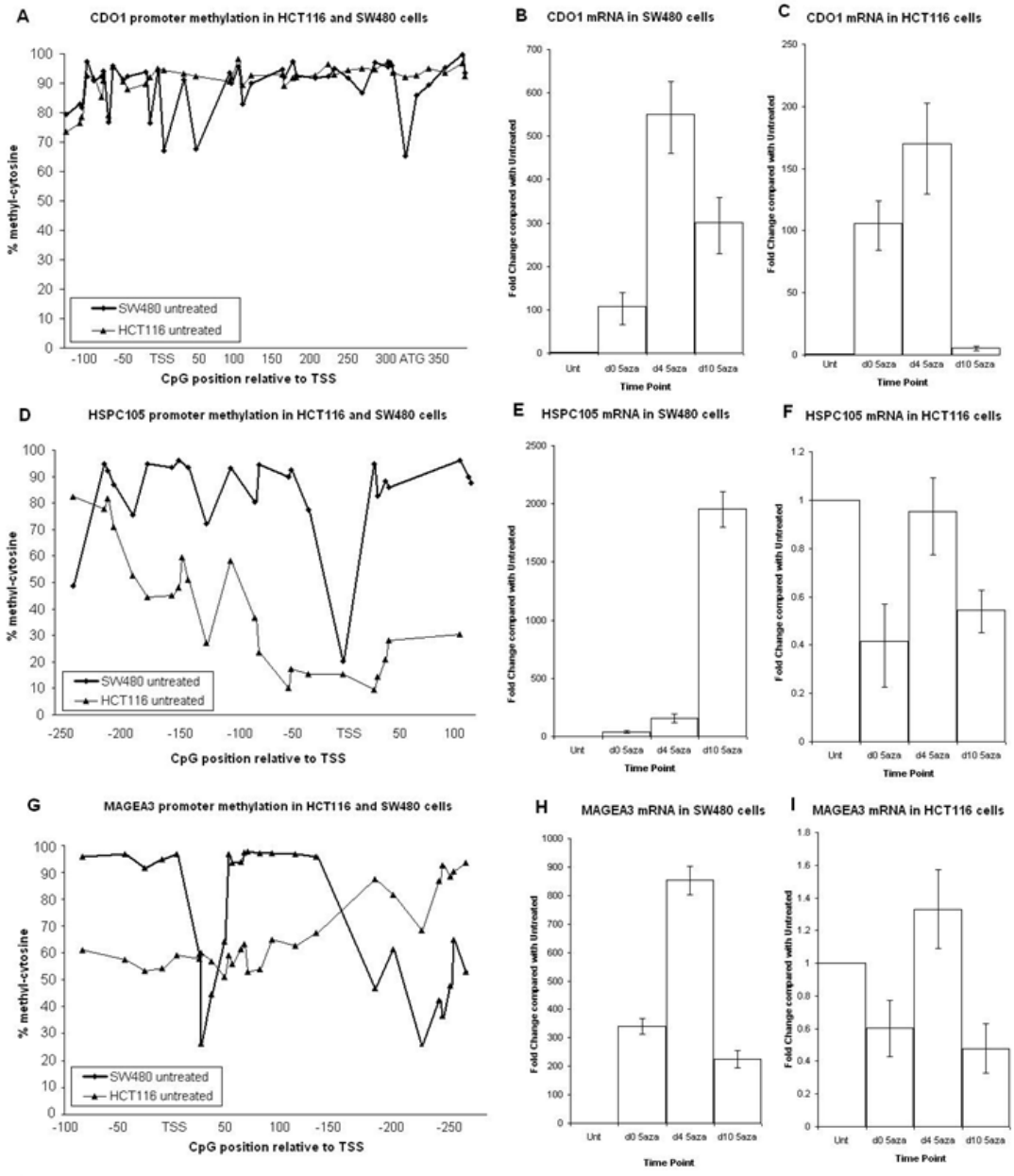
J

Gene	Cell Line	Expression Pattern	Histone Modification							
			H3Ac		H3K4me3		H3K9me3		H3K27me3	
			d0	d10	d0	d10	d0	d10	d0	d10
CXCL6	HCT116 SW480	Short term expressed Always expressed		●	●	● ●	●			
CDKN2A	HCT116 SW480	Always expressed Short term expressed	●		●	● ●	●	v	v	v v
ZFP3	HCT116 SW480	Short term expressed Up-regulated	 ●	 ●	●	v ●	 ●	v	●	

Key:
● = Significantly increased
v = Significantly decreased

Figure 2 – CpG island methylation of short term reactivated genes with respect to different expression types. A – *CXCL6* CpG island methylation; short term v

always-expressed**.** SW480 displayed hypomethylation and was expressed at all time points **(B)**.Uniform hypermethylation of the HCT116 cell line was associated with a short term reactivation of expression **(C)**. **D** – *CDKN2A* CpG Island methylation; short term v monoallelic expression SW480 cells display hypermethylation and were temporarily re-expressed **(E)**, whilst HCT116 are ~50% methylated at CpG sites near the TSS, and were expressed at all time points **(F)**. **G** – *ZFP3* CpG Island methylation; short term v lowly expressed. SW480 cells showed hypomethylation at CpG sites near the TSS and expression was up-regulated after 5-aza-dC treatment **(H)**. HCT116 remains hypermethylated and was temporarily reactivated with 5-aza-dC **(I)**. **J** – Significant changes to histone modifications compared with untreated cells ($p<0.05$).



J

Gene	Cell Line	Expression Pattern	Histone Modification							
			H3Ac		H3K4me3		H3K9me3		H3K27me3	
			d0	d10	d0	d10	d0	d10	d0	d10
CDO1	HCT116	Up-regulated			●	●		●	v	v
	SW480	Long term re-expressed		●		●	●	●	v	v
HSPC105	HCT116	Always expressed	●	●	v		●			v
	SW480	Long term re-expressed	●	●	●					
MAGEA3	HCT116	Always expressed		●	●		●	v	v	v
	SW480	Long term re-expressed		●	●	●	●		v	v

Key:
● = Significantly increased
v = Significantly decreased

Figure 3 – Promoter CpG Island methylation of long term reactivated genes. A – *CDO1* CpG Island methylation; long term v short term expressed. Sequencing

analysis revealed CpG sites near the TSS in SW480 cells have lower methylation and long term expression **(B)** compared with HCT116 cells which are uniformly hypermethylated and short term expressed **(C). D** *HSPC105* CpG Island methylation; always-expressed v long term expressed. The SW480 cell line shows localised hypomethylation at the TSS and can remain expressed ten days post treatment **(E)**. The HCT116 cell line is hypomethylated at the *HSPC105* promoter and is continually expressed **(F)**. **G** – *MAGEA3* CpG Island methylation; always-expressed v long term re-expressed. SW480 cells show localised hypomethylation at the TSS and are expressed ten days post treatment **(H).** HCT116 cells show ~50% methylation at the TSS and MAGEA3 is expressed at all time points **(I)**. **J** – Significant changes to histone modifications compared with untreated cells ($p<0.05$).

Genes determined to be 'always-expressed' displayed a maximum of 50% methylation at the TSS which is indicative of mono-allelic methylation, and up-regulated genes displayed varying patterns of methylation. The *MLH1* gene was expressed in both cell lines and methylation was not detected in the TSS associated CpG island. *CDKN2A* was expressed in HCT116 and showed partial methylation at the TSS. The corresponding region in SW480 cells was hypermethylated and expression was classified as short term reactivated after treatment.

Continued incubation of cells in drug free media allowed re-methylation, which returned to pre-treatment levels at promoter CpG islands. Gene expression was not necessarily affected by the return of promoter methylation however, and expression of the *CDO1*, *HSPC105* and *MAGEA3* genes in SW480 cells remained high ten days after 5-aza-dC treatment. These genes displayed unique CpG island methylation patterns that feature hypomethylated CpG sites adjacent to the TSS. As methylation levels at promoter CpG islands did not accurately reflect the expression of the genes

studied, we sought to examine the patterns of histone modifications at the respective CpG islands that may account for high levels of expression.

Chromatin modifications with 5-aza-dC

Chromatin Immunoprecipitation and q-PCR revealed that Histone H3Ac and H3K4me3 were features associated with expressed genes such as *GAPDH* and *MLH1* and repressed genes were associated with H3K9me3 and less frequently H3K27me3. Repressive marks were absent from constitutively expressed genes, however H3K4 trimethylation which is commonly associated with activated genes was also present in genes that were not actively transcribed. ChIP results are summarised in Figure 2 and 3, with p-values listed in Table 2. Specific changes in chromatin modification are shown in Supplementary figures 1-4.

Following 72 h of exposure to 5-aza-dC, increases of H3Ac, H3K4me3 and H3K9me3 were observed. Decreases in H3K27me3 were observed when the feature was present in untreated cells. Interestingly H3K9me3 increased after treatment; however this level receded over the next ten days regardless of the level of resulting gene expression. A comparison of histone modifications in untreated and ten days post treated cells at short term reactivated genes revealed a significant increase of histone H3K4me3 and decreased level of trimethyl-histone H3 lysine 9 and 27. Despite this, transcription of these genes ten days post treatment was at a similar to untreated cells. The single most apparent difference between short and long term reactivated genes was the H3Ac modification. Genes deemed to be 'long term reactivated' revealed an increase of H3Ac after treatment which persisted until ten days after drug treatment. Long-term expressed genes also experienced a reduction in the repressive H3K27me3 when present in untreated cells. Histone H3Ac and

H3K4me3 levels at genes which were always-expressed or those which were up-regulated were typically stable or increased slightly.

Table 2: P-values for changes to histone modifications in comparison to untreated cells.

Gene	Cell line	H3Ac		H3K4me3		H3K9me3		H3K27me3	
		d0	d10	d0	d10	d0	d10	d0	d10
CXCL6	**HCT116**	0.163	0.081	0.010	0.010	0.047	0.173	0.122	0.368
	SW480	0.499	0.008	0.231	0.005	0.829	0.622	0.565	0.656
CDKN2A	**HCT116**	0.005	0.230	0.028	0.029	0.014	0.005	0.006	0.001
	SW480	0.478	0.063	0.220	0.024	0.639	0.117	0.078	0.004
ZFP3	**HCT116**	1.000	0.194	0.033	0.006	1.000	0.000	0.036	1.000
	SW480	0.008	0.007	0.300	0.005	0.008	0.074	0.286	0.061
CDO1	**HCT116**	0.141	0.153	0.004	0.008	0.531	0.026	0.020	0.004
	SW480	0.066	0.006	1.000	0.005	0.007	0.047	0.003	0.001
HSPC105	**HCT116**	0.004	0.018	0.008	0.108	0.004	0.377	0.225	0.001
	SW480	0.031	0.016	0.046	0.069	0.243	0.068	0.286	0.368
MAGEA3	**HCT116**	0.092	0.030	0.022	0.066	0.001	0.001	0.013	0.015
	SW480	0.368	0.013	0.039	0.033	0.012	0.232	0.016	0.011

Discussion

The epigenetic control of gene expression is mediated by DNA methylation and histone modifications. By altering the DNA methylation and up-regulating gene expression we can identify patterns of change in histone protein modifications that accompany the long and short term reactivation of epigenetically silenced genes. Of particular relevance to this study was the apparent transcriptional up-regulation of silenced genes that displayed minimal demethylation, where histone modifications are likely to be involved in the regulation of gene expression in these instances.

The effect of DNA methylation on gene expression

Regardless of the expression pattern during drug treatment, the extent of methylation of particular CpG islands remained relatively unchanged compared to genomic levels after 5-aza-dC exposure. This observation has been made previously, with methylation of repeat sequences likely to contribute to this discrepancy [19, 20]. Constitutively expressed genes were hypomethylated on both alleles, or exhibited CpG island methylation of 50% indicating mono-allelic methylation, such as the *CDKN2A* gene in HCT116 cells. Slight demethylation of promoter CpG islands was induced with 5-aza-dC, yet expression was not necessarily restricted in some genes when the methylation returned to pre-treatment levels. High expression of long term reactivated genes appeared to be dependent upon pre-existing hypomethylation at the TSS regardless of whether the adjacent CpG sites were hypermethylated. This result indicates the pattern of methylation rather than the general level of methylation across the CpG island is crucial to re-activating silenced genes via interactions with other epigenetic factors. Hypomethylated CpG sites within hypermethylated promoters have been identified previously in the Oncostatin M receptor gene [21] but the effect of this hypomethylation on transcription was not examined. Why expression of the long term reactivated genes did not occur in the untreated cells which displayed a near identical methylation pattern may be explained by changes in the modifications on the histone proteins.

The effect of histone modifications on gene expression

A snapshot of factors governing gene expression was achieved with the compilation of CpG island sequencing and chromatin immuno-precipitation results. Upon the reactivation of numerous genes and classification of expression, we could distinguish

between the genes based on the methylation and chromatin changes present. These generalisations are summarised in Table 1. Prior to treatment, transcriptionally inactive genes were characterised by higher levels of repressive modifications and lower levels of activating marks. Upon treatment with 5-aza-dC there was generally an increase to the levels of H3K4me3 and H3K9me3, whilst H3Ac increased only in some of the genes. Reductions to H3K27me3 occurred in genes that initially displayed this trait. With regards to chromatin modifications, it was during the drug free growth period that histone acetylation became the most distinguishable feature between short and long term reactivated genes. Long term reactivated gene promoters became increasingly associated with acetylation of histone H3 assisting with gene activation. Temporarily reactivated genes did not attract this modification despite a brief period of expression. It would therefore appear that the introduction of H3 acetylation is a crucial factor in reversing transcriptional status of an epigenetically silenced gene which is assisted by DNA hypomethylation. With the exception of an increase in H3K4 trimethylation, long-lasting changes to epigenetic modifications were generally not observed in the temporarily reactivated genes.

The up-regulation of lowly expressed genes was associated with increased H3 acetylation and H3K4me3, such as the *ZFP3* gene in SW480 cells. Methylation profiles such as this may indicate an intermediate between always-expressed genes and the long term reactivated genes. Co-existing active and repressive marks may allow a restricted level of transcription, suggesting the control of expression of these genes is dependent on equilibrium of both types of modifications.

In the genes examined in this study, the roles of histone H3 acetylation and H3K27me3 were apparent as activating and repressing marks respectively, however the function of H3K4me3 and H3K9me3 were not as clearly linked to their proposed

functions. Following 5-aza-dC exposure, H3K9me3 was frequently increased at expressed genes which concurs with recent findings that it can also be coupled with gene activation [20, 22, 23]. Similarly, H3K4me3 which associates with active regions of the genome was found at inactive genes, albeit at a reduced level. Observations of this nature highlight the dynamic nature of chromatin and possibly suggest an intermediate form of repression similar to bivalent chromatin surrounding developmental genes [24].

Linking DNA methylation, chromatin modifications and gene expression

The patterns of expression of re-activated and up-regulated genes can be largely explained with the combination of promoter methylation analysis and chromatin immuno-precipitation assays (and are outlined in Table 1). By observation and comparison of long and short term reactivated genes, our results show that genes with a localised hypomethylation at the TSS can more readily facilitate an increase of histone H3 acetylation than genes undergoing demethylation by 5-aza-dC. Additionally we observed that expression was reactivated without major change in the CpG island methylation and this was independent of nearby hypermethylation within the same CpG island as the TSS. Following 5-aza-dC treatment localised hypomethylation is capable of initiating alteration of the chromatin structure to allow transcription.

A sequence of events involved with epigenetic reactivation has been proposed by Litt *et al.* [25]. The authors state that reactivation of the *HPRT* gene required hemi-demethylation of the promoter, 'opening' of chromatin structure, transcription factor binding and assembly of the transcription complex prior to synthesis of the *HPRT* RNA. Based on our experiments, we can extend on this knowledge by suggesting that

minor demethylation induced by 5-aza-dC and increased H3K4me3 allows initiation of transcription. The role of H3K9 trimethylation is not clear but may be associated with reactivation in some instances. A loss of repressive marks such as H3K27me3 can also further increase transcription. Although not proven, it is possible MBD2 binding could be lost at this point which would no longer deter histone acetyltransferase from the region [26]. Transcription is prolonged if increased acetylation of histone H3 occurs, otherwise expression is transient and gene expression is likely to revert to an inactive state.

Methyltransferase Inhibitors in treatment of tumours

The efficacy of 5-aza-dC as a treatment is limited to certain tumour types, however, what causes a favourable outcome from 5-aza-dC treatment is unknown. Its success may lie with the methylation pattern at currently un-identified target genes and the drugs ability to reactivate silenced genes over a longer period of time. Therefore, our observations would support the use of combined methyltransferase / histone deacetylase inhibitors in the treatment of malignancy. Histone deacetylase inhibitors may increase acetylation at hypermethylated transcription start sites leading to long term reactivation of anti-proliferative genes.

Analysis of the chromatin at the promoter of the genes in this study suggests that existing hypomethylation following (but not necessarily induced by) 5-aza-dC treatment, attracts histone H3 acetylation. The combination of hypomethylation of CpG sites at the TSS and H3 acetylation result in stable gene reactivation. The results of this study highlight the epigenetic features which need to be modified with regard to the reversal of transcriptional status of genes in the treatment of disease. A more

strategic approach will lead to the development of epigenetic therapies rather than use of epigenetic-modifying drugs as cytotoxic therapies.

Methods

Cell Culture

HCT116 and SW480 cells were grown in DMEM media supplemented with 10% foetal calf serum (Sigma-Aldrich, St Louis, MO, USA) at 37 ºC and 5% CO_2. Cells were treated with 5-aza-2'-deoxycytidine (Sigma-Aldrich) as previously described [19]. DNA and RNA were extracted from untreated cells, 5-aza-dC treated cells (72 h of treatment), and at 4 and 10 days after cessation of treatment (Day 4 and 10 of re-methylation). The cells were originally obtained from the ATCC and were authenticated using the Identifiler DNA identification kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions.

Global methylation

Global methylation was assessed as described previously [19]. Briefly, 50 µg of DNA was enzymatically digested with Nuclease P1 (US Biological, Swampscott, MA, USA) followed by chromatographic separation on a Varian Star Chromatography workstation with a Supelcosil LC-18-DB column (Sigma-Aldrich). Absorbance was monitored at 278 nm and peak areas were quantified with Star Reviewer Software (Varian, Palo Alto, CA, USA). The 5-methylcytosine content was expressed as a percentage of the total cytosine pool after correction for extinction co-efficients.

Bisulfite Sequencing

DNA was converted in duplicate using a Qiagen Epitect Bisulfite conversion kit (Qiagen, Valencia, CA, USA) using 2 µg of phenol-chloroform purified DNA. Samples were eluted in 30 µL of elution buffer and an aliquot was diluted 1:3 prior to PCR and stored at 4 ºC, whilst the remaining fraction was stored at -20ºC. CpG islands surrounding the transcription start site of genes were targeted in PCR analysis using the primers listed in Supplementary Table 1. Sequencing reactions were performed in duplicate and were analysed on an ABI 3730 sequencer. Data analysis was carried out using Sequence Scanner software (Applied Biosystems). The percentage methylation at each CpG was determined by dividing the cytosine peak by the combined heights of the cytosine and thymine peaks as described previously [21].

Real Time PCR Analysis of gene expression

RNA was converted to cDNA using Superscript II (Invitrogen) and random primers (Promega) according to manufacturer's instructions. Reactions were performed in triplicate using primers listed in Supplementary Table 1, 2X SYBR Green (Applied Biosystems) in an ABI PRISM 7500 PCR machine (Applied Biosystems). C_T values were determined automatically by Sequence Detection Software version 1.4 and final calculations were expressed as fold differences compared to β-actin using the $\Delta\Delta C_T$ method. Genes with undetected expression were assigned a C_T value of 40.

Chromatin Immunoprecipitation (ChIP) and Analysis

Briefly, the crosslinking of DNA with protein and cell lysis was performed using the EZ-Magna ChIP A Kit (Upstate/Millipore) according to the manufacturer's instructions. Sonication was performed using 8 x 30-second cycles at 60% duty cycle in an ice bath and samples were further cooled for 30 seconds in between sonication cycles. Chromatin Immunoprecipitation was performed as previously described [27] with slight modifications. Antibodies used were all obtained from Upstate with the exception of the Rabbit IgG non-specific antibody from Santa Cruz Biotechnology. Antibody quantities per reaction were determined in preliminary experiments and were 5 µL for α-acetyl H3, 5 µL for αH3K4me3, 4 µL for αH3K9me3, 4 µL for αH3K27me3 and 5 µL of Rabbit IgG was added to negative control samples. Cross links were reversed with the addition of 20 µL Proteinase K (Promega) and incubated at 62°C for 3 h with shaking. Recovered DNA was then purified using a PCR clean up kit (Qiagen, Valencia, CA, USA).

Real time PCR analysis of immunoprecipitated chromatin

DNA was quantified using the DNA Quantitation System (Promega) according to the manufacturer's instructions, and measurements were taken using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). The real-time PCR reactions were performed using 200 ρg of DNA template with SYBR Green 2X mastermix (Applied Biosystems) and primers listed in Supplementary Table 1. Reactions were performed in triplicate and carried out using an ABI PRISM 7500 PCR machine (Applied Biosystems). C_T values were determined automatically by Sequence Detection Software version 1.4 (Applied Biosystems) and the final values were expressed as a percentage of the input fraction.

Statistical Analysis

Standard deviations were calculated and a T-test was employed to compare expression levels and histone modification levels in drug treated cells against untreated cells. *P*-values less than 0.05 were considered to be statistically significant.

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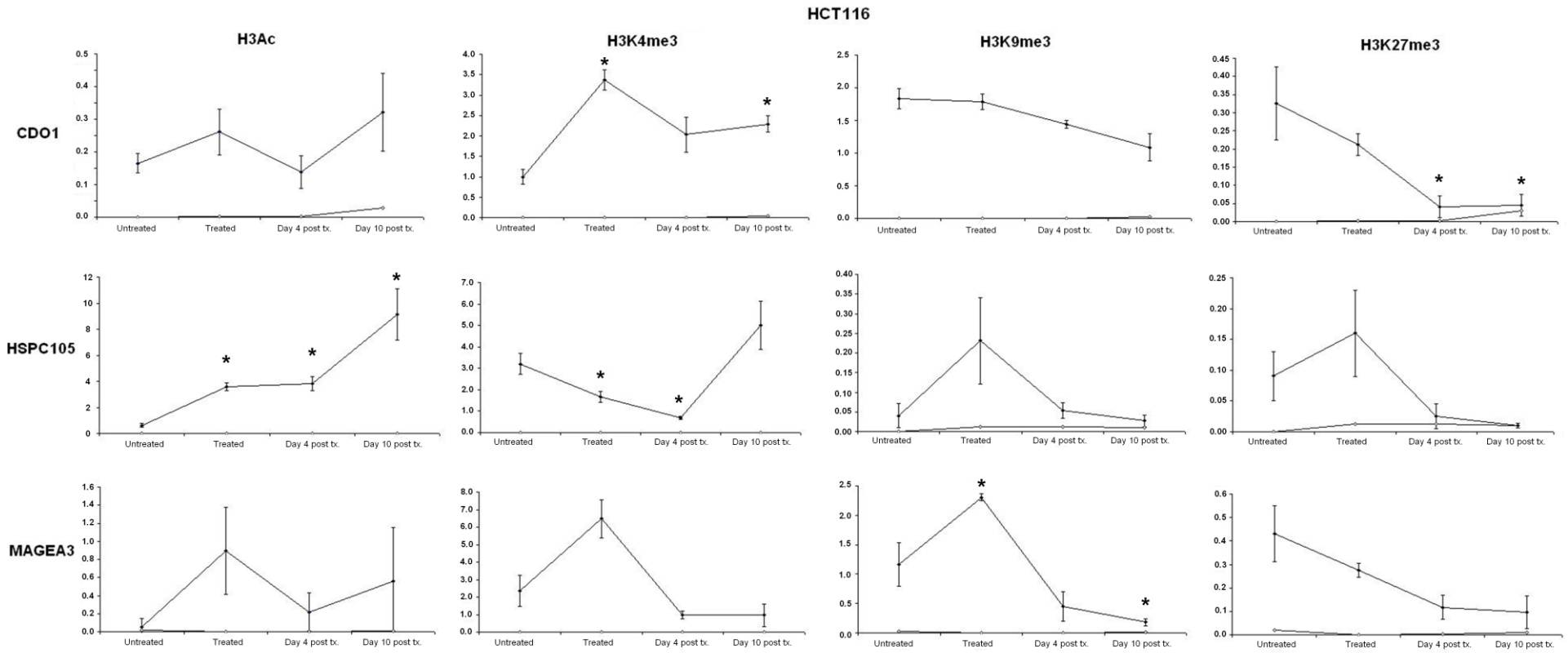
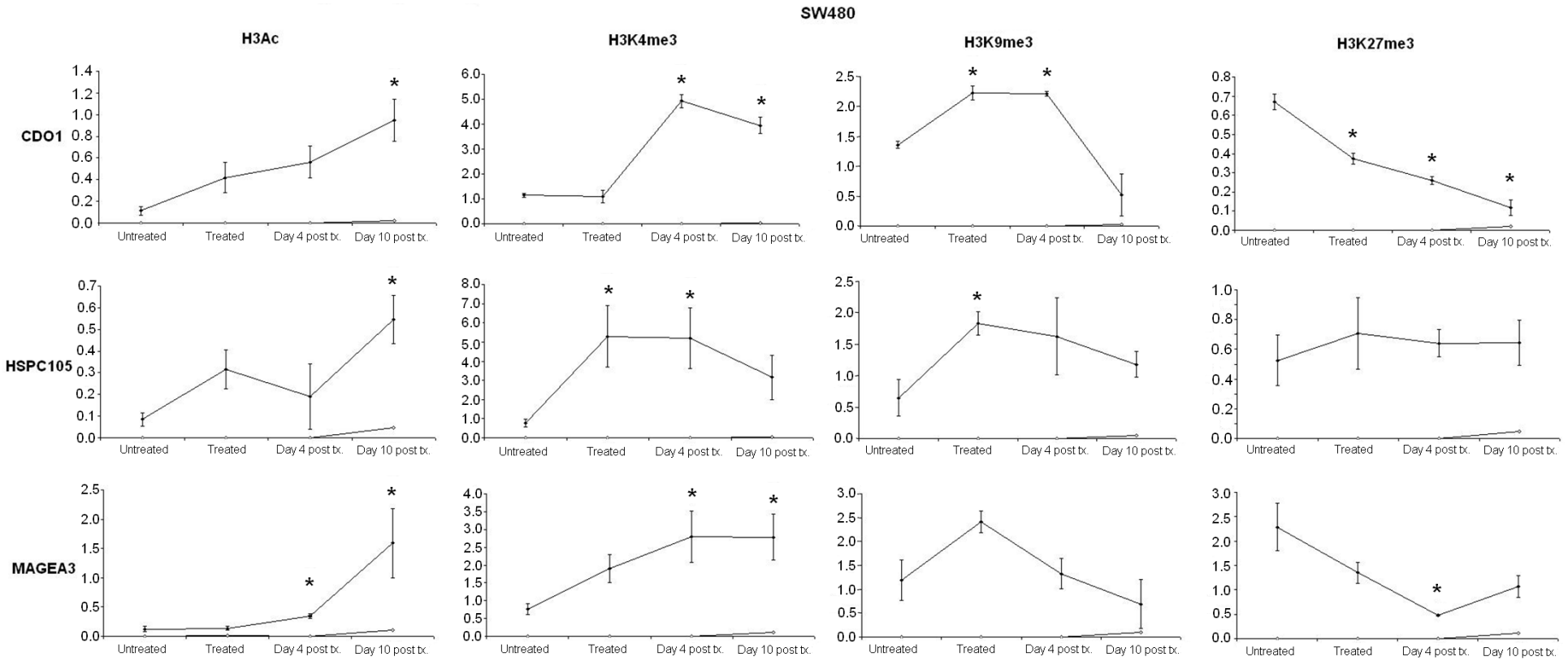
References

1. Luger K, Mader A W, Richmond R K, Sargent D F, and Richmond T J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature; 389(6648): p. 251-60.
2. Owen-Hughes T and Workman J L. (1994) Experimental analysis of chromatin function in transcription control. Crit Rev Eukaryot Gene Expr; 4(4): p. 403-41.
3. McGhee J D and Felsenfeld G. (1980) Nucleosome structure. Annu Rev Biochem; 49: p. 1115-56.
4. Lee D Y, Hayes J J, Pruss D, and Wolffe A P. (1993) A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell; 72(1): p. 73-84.
5. Zhang Y and Reinberg D. (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev; 15(18): p. 2343-60.
6. Kwon M J, Kim S S, Choi Y L, Jung H S, Balch C, et al. Derepression of CLDN3 and CLDN4 during Ovarian Tumorigenesis Is Associated with Loss of Repressive Histone Modifications. Carcinogenesis; 2010: p. 6.
7. Wu H, Min J, Lunin V V, Antoshenko T, Dombrovski L, et al. Structural biology of human H3K9 methyltransferases. PLoS One; 5(1): p. e8570.
8. Belinsky S A, Nikula K J, Palmisano W A, Michels R, Saccomanno G, et al. (1998) Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci U S A; 95(20): p. 11891-6.
9. Mittag F, Kuester D, Vieth M, Peters B, Stolte B, et al. (2006) DAPK promotor methylation is an early event in colorectal carcinogenesis. Cancer Lett; 240(1): p. 69-75.
10. Futscher B W, O'Meara M M, Kim C J, Rennels M A, Lu D, et al. (2004) Aberrant methylation of the maspin promoter is an early event in human breast cancer. Neoplasia; 6(4): p. 380-9.
11. Koga T, Takeshita M, Yano T, Maehara Y, and Sueishi K. CHFR hypermethylation and EGFR mutation are mutually exclusive, and exhibit contrastive clinical backgrounds and outcomes in non-small-cell lung cancer. Int J Cancer; 2010: p. 12.
12. You Y, Ma L, You M, Li X, Wang S, et al. TSLC1 gene silencing in cutaneous melanoma. Melanoma Res; 20(3): p. 179-83.
13. Ramos E A, Camargo A A, Braun K, Slowik R, Cavalli I J, et al. Simultaneous CXCL12 and ESR1 CpG island hypermethylation correlates with poor prognosis in sporadic breast cancer. BMC Cancer; 10(23): p. 23.
14. Ooki A, Yamashita K, Kikuchi S, Sakuramoto S, Katada N, et al. Potential utility of HOP homeobox gene promoter methylation as a marker of tumor aggressiveness in gastric cancer. Oncogene; 29(22): p. 3263-75.
15. Baba Y, Nosho K, Shima K, Freed E, Irahara N, et al. (2009) Relationship of CDX2 loss with molecular features and prognosis in colorectal cancer. Clin Cancer Res; 15(14): p. 4665-73.

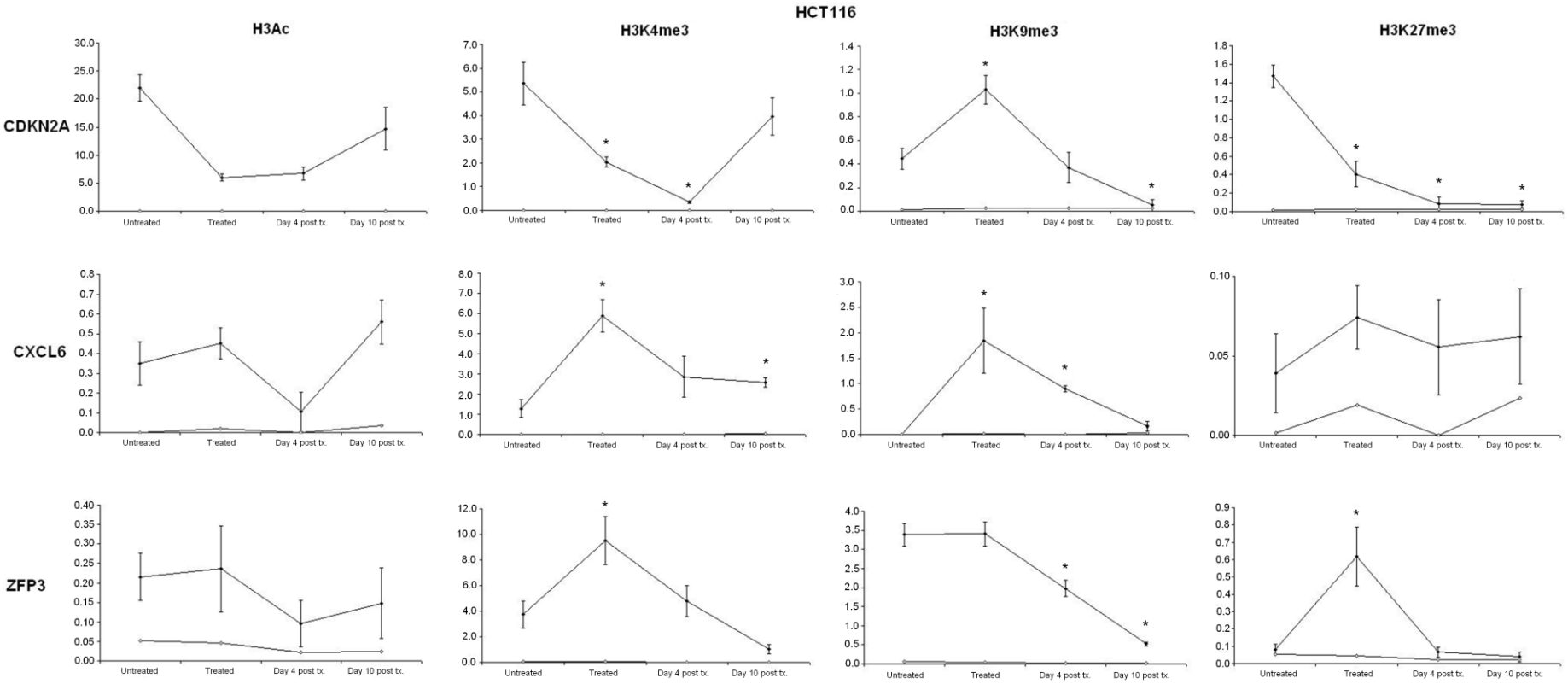
16. Hitchins M P, Wong J J, Suthers G, Suter C M, Martin D I, et al. (2007) Inheritance of a cancer-associated MLH1 germ-line epimutation. N Engl J Med; 356(7): p. 697-705.
17. Kuiper R P, Ligtenberg M J, Hoogerbrugge N, and Geurts van Kessel A. Germline copy number variation and cancer risk. Curr Opin Genet Dev; 20(3): p. 282-9.
18. Aparicio A and Weber J S. (2002) Review of the clinical experience with 5-azacytidine and 5-aza-2'-deoxycytidine in solid tumors. Curr Opin Investig Drugs; 3(4): p. 627-33.
19. Mossman D, Kim K T, and Scott R J. (2010) Demethylation by 5-aza-2'-deoxycytidine in colorectal cancer cells targets genomic DNA whilst promoter CpG island methylation persists. BMC Cancer; 10(1): p. 366.
20. Lim H W, Iwatani M, Hattori N, Tanaka S, Yagi S, et al. (2009) Resistance to 5-aza-2'-deoxycytidine in Genic Regions Compared to Non-genic Repetitive Sequences. J Reprod Dev; 9: p. 9.
21. Deng G, Kakar S, Okudiara K, Choi E, Sleisenger M H, et al. (2009) Unique methylation pattern of oncostatin m receptor gene in cancers of colorectum and other digestive organs. Clin Cancer Res; 15(5): p. 1519-26.
22. Vakoc C R, Mandat S A, Olenchock B A, and Blobel G A. (2005) Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. Mol Cell; 19(3): p. 381-91.
23. Wiencke J K, Zheng S, Morrison Z, and Yeh R F. (2008) Differentially expressed genes are marked by histone 3 lysine 9 trimethylation in human cancer cells. Oncogene; 27(17): p. 2412-21.
24. Bernstein B E, Mikkelsen T S, Xie X, Kamal M, Huebert D J, et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell; 125(2): p. 315-26.
25. Litt M D, Hansen R S, Hornstra I K, Gartler S M, and Yang T P. (1997) 5-Azadeoxycytidine-induced chromatin remodeling of the inactive X-linked HPRT gene promoter occurs prior to transcription factor binding and gene reactivation. J Biol Chem; 272(23): p. 14921-6.
26. Magdinier F and Wolffe A P. (2001) Selective association of the methyl-CpG binding protein MBD2 with the silent p14/p16 locus in human neoplasia. Proc Natl Acad Sci U S A; 98(9): p. 4990-5.
27. Mitchell C M, Johnson R F, Giles W B, and Zakar T. (2008) Prostaglandin H synthase-2 gene regulation in the amnion at labour: histone acetylation and nuclear factor kappa B binding to the promoter in vivo. Mol Hum Reprod; 14(1): p. 53-9.



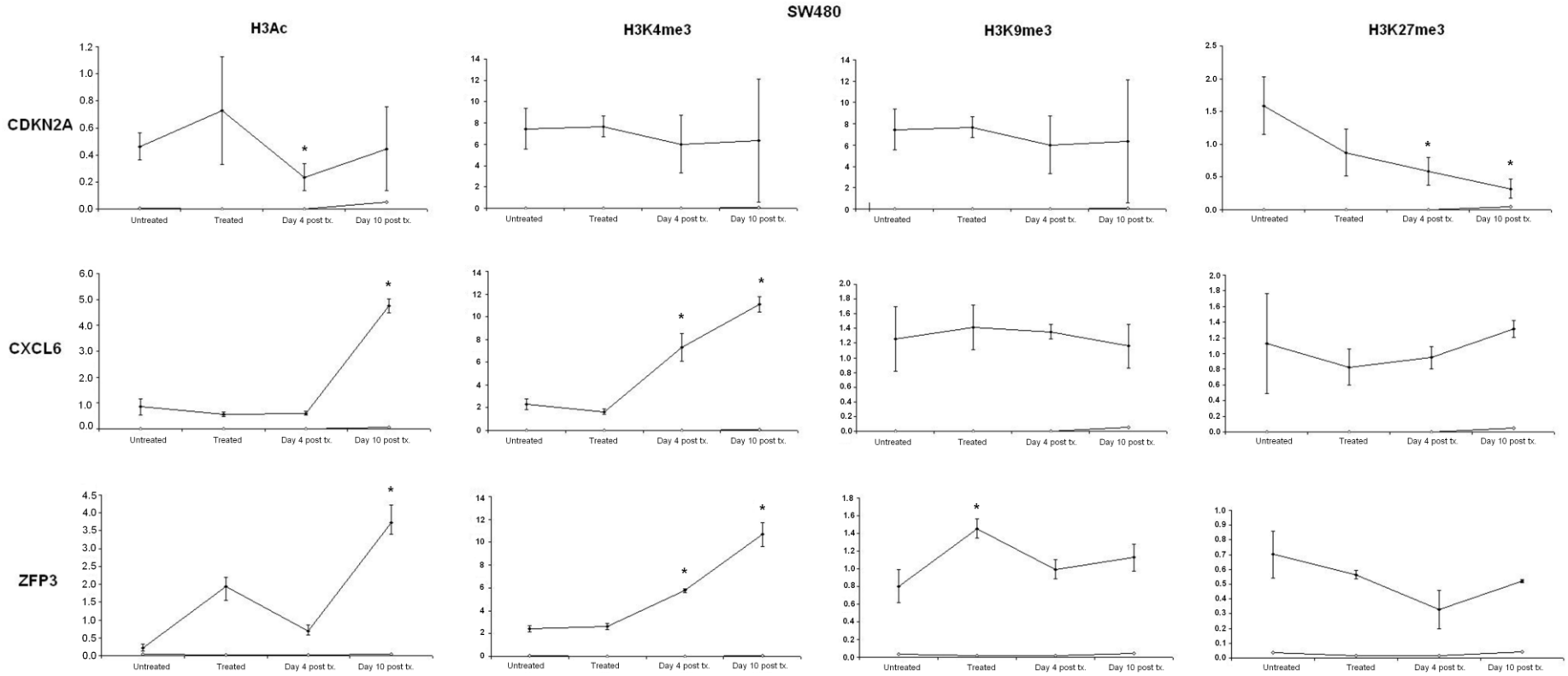
Supplementary Figure 1 - HCT116 chromatin changes at *CDO1*, *HSPC105* and *MAGEA3* transcription start sites. Mock treatments are plotted with white data points and 5-aza-dC treated cells are plotted with black data points. The y-axis values represent percentage input, whilst x-axis values represent the four time-points of cell culture.



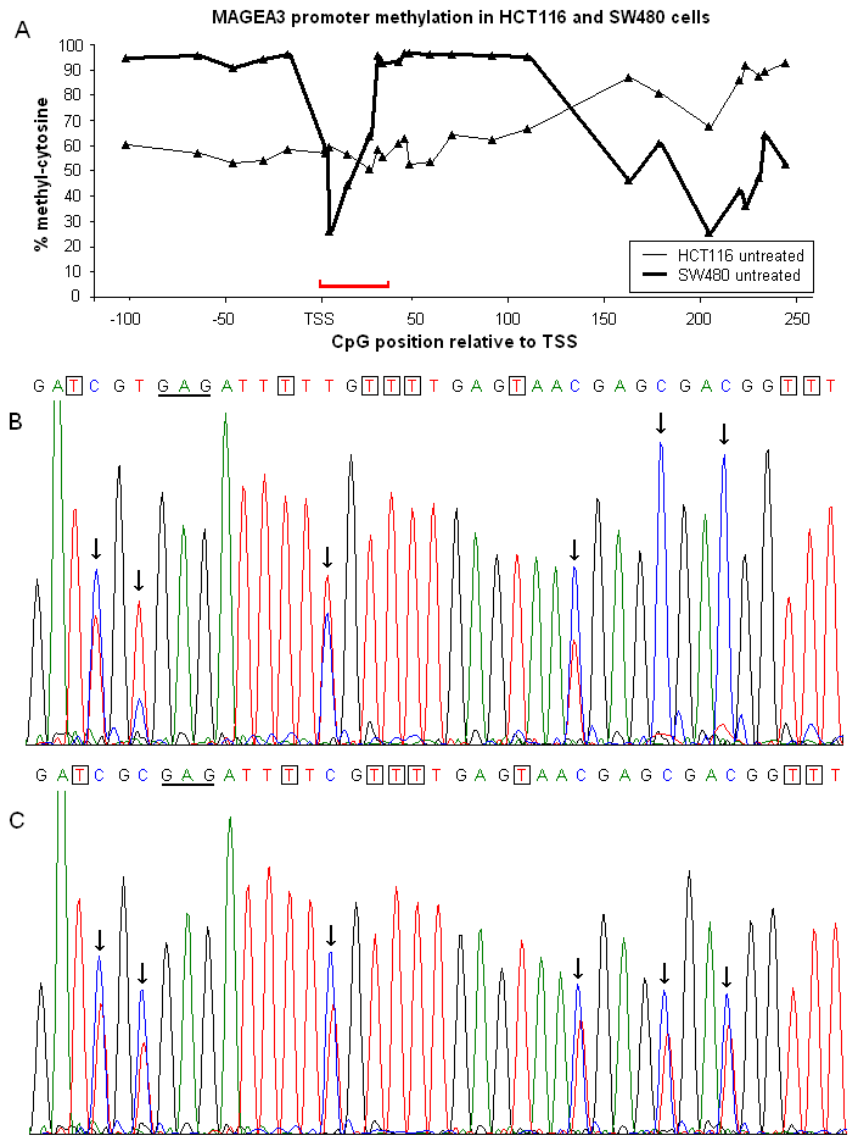
Supplementary Figure 2 – SW480 chromatin changes at CDO1, HSPC105 and MAGEA3 transcription start sites. Mock treatments are plotted with white data points and 5-aza-dC treated cells are plotted with black data points. The y-axis values represent percentage input, whilst x-axis values represent the four time-points of cell culture.



Supplementary Figure 3 – HCT116 chromatin changes at *CDKN2A*, *CXCL6* and *ZFP3* transcription start sites. Mock treatments are plotted with white data points and 5-aza-dC treated cells are plotted with black data points. The y-axis values represent percentage input, whilst x-axis values represent the four time-points of cell culture.



Supplementary Figure 4 – SW480 chromatin changes at CDKN2A, CXCL6 and ZFP3 transcription start sites. Mock treatments are plotted with white data points and 5-aza-dC treated cells are plotted with black data points. The y-axis values represent percentage input, whilst x-axis values represent the four time-points of cell culture.



Supplementary Figure 5 – Methylation at the MAGEA3 Transcription Start Site. A - Promoter methylation across the MAGEA3 CpG island. The red bar indicates the region of sequence shown in B and C. **B** – Methylation at the MAGEA3 TSS in SW480 cells. Direct sequencing of bisulfite PCR products causes dual C and T peaks at CpG sites, and are representative of methylated and non-methylated alleles respectively. The CpG site adjacent to the TSS shows a greater proportion of T alleles (representing unmethylated cytosine) indicating hypomethylation, while nearby CpG sites show increased cytosine peaks and higher methylation levels. Arrows indicate CpG sites, boxed T's indicate position of non-CpG cytosine and underlined sequence represents the transcription start site. **C** – CpG sites in the HCT116 cell line are greater than 50% methylated.

Supplementary Table 1 – Primer sequences used in Bisulfite PCR / ChIP / qPCR.

Gene (Accession Number)	Primer Direction	Bisulfite PCR and Sequencing (5'>3') (Relative to transcription start site)	ChIP (5'>3') (Based on accession number)	Expression qPCR (5'>3') (Based on accession number)
GAPDH (NM_002046.3)	Forward	GTTGGGATTGGTTGAGTT (-71,-53)	TACTAGCGGTTTTACGGGCG (-118-99)	N/A
	Reverse	CCAAACCTCCATACCCAAC (225,247)	TCGAACAGGAGGAGCAGAGAGCGA (25-48)	N/A
ACTB (NM_001101.2)	Forward	N/A	TGTGGCATCCACGAAACTACC (887- 907)	TGTGGCATCCACGAAACTACC (887,-907)
	Reverse	N/A	ACATCTGCTGGAAGGTGGACA (1119-1139)	ACATCTGCTGGAAGGTGGACA (1119-1139)
CDO1 (NM_001801.2)	Forward	TTAAAGTGGGGGAGAGATTG (-192, -172)	GAGGGAAAACCAGTGTGCCTAC (686-707)	GGGAAAACCAGTGTGCCTACATC (688-710)
	Reverse	AACCTACACCTCCTCTACATTA (391, 413)	GCTCACAGCAGGTTCCGTATG (750-770)	GTACAAGTGAAGGCTCACAGC (762-782)
HSPC105 (NM_145168.2)	Forward	GTGAAAGTTTAAAAGTAGATAT (-282, -260)	GTGTCCTCATTACAGGAGG (157-175)	GTGTCCTCATTACAGGAGG (157-175)
	Reverse	CATTCTAAAAAACCAAACTAC (279, 300)	GCTTTCTCTACGTCAGACAGG (308-328)	GCTTTCTCTACGTCAGACAGG (308- 328)
MAGEA3 (NM_005362.3)	Forward	GGATTTATAGTTTTAGGAT (-188, -170)	ATCTGCCAGTGGGTCTCCATT (141-161)	ATCTGCCAGTGGGTCTCCATT (141-161)
	Reverse	CACATTAAACTCTATCCCCAAAA (242, 265)	TCTGCTCAAGAGGCATGATGA (205-225)	TCTGCTCAAGAGGCATGATGA (205- 225)
CXCL6 (NM_002993.3)	Forward	AGGGATGAATGTAGATAAAGGGAGTGT (-62,-35)	AGCTCAGGAACCCGCGAAC (162-180)	AACCCCAAAACGATTGGTAAACT (370- 392)
	Reverse	CTTACACCACTTCCACCTTAA (382,403)	CAGTGCCAGGAGCTCTCAC (305-323)	GACAAACTTGCTTCCCGTTCTT (448- 469)
ZFP3 (NM_153018.1)	Forward	GAGTTTTTGAGTTTAGAGTAATGT (-330, -306)	CTTCGGGCAGAGTTCTGAGC (1076-1095)	CTTCGGGCAGAGTTCTGAGC (1076-1095)
	Reverse	CATAAACTTCAAAATCACACAAC(223, 246)	CTGAGTTCCCCCTGAAGGCC (1241-1260)	CTGAGTTCCCCCTGAAGGCC (1241-1260)
CDKN2A (NM_000077.3)	Forward	GATTTTAGGGGTGTTAT (-46, -29)	GTCGGAGGCCGATCCAGGTCATGA (346-369)	GTCGGAGGCCGATCCAGGTCATGA (346-369)
	Reverse	CTCATTCCTCTTCCTTAAC (333, 352)	AGCGTGTCCAGGAAGCCCTC (474-493)	AGCGTGTCCAGGAAGCCCTC (474-493)
MLH1 (NM_000249.2)	Forward	AGATTATTTTAGTAGAGG (314, 296)	AGCTGATGGAAAGTGTGCATACA (417-439)	AGCTGATGGAAAGTGTGCATACA (417-439)
	Reverse	AAAAAACCTAACTAACA (317, 334)	CGTGATCTGGGTCCCTTGA (495-513)	CGTGATCTGGGTCCCTTGA (495-513)
DICER1 (NM_030621.2)	Forward	N/A	N/A	CAAACCAGGTTGCTCAACAAG (490-510)
	Reverse	N/A	N/A	AACCTTGAGATCTGAATGAGTTCTGA (519-545)

Chapter 6

General Discussion

This series of experiments was performed with the broad aim of gaining a greater understanding of the mechanisms involved in epigenetic inactivation and regulation of gene expression in colorectal cancer. In particular this work is an examination of how gene expression is altered in response to 5-aza-2-deoxycytidine and Trichostatin A exposure and which epigenetic features are reversible. A study was also undertaken to investigate the role of a polymorphism within the Δ*DNMT3B* gene as a modifier of the age of disease onset via an epigenetic mechanism.

At the commencement of this study, there were increasing amounts of evidence indicating that epigenetic repression of transcription mirrored diverse phenotypes that were comparable to mutation carriers of respective disease genes. An emerging issue was the phenomenon of inherited epimutations. Epigenetic events are thought to be heritable events in the context of cellular division, however there is new evidence demonstrating trans-generational inheritance of epimutations [116, 119]. These rare events may lead to mosaic or soma-wide inactivation of one allele, and may potentially account for a small proportion of cases of HNPCC. The mechanism with which an epimutation is established remains unknown and is the subject of much interest. Currently this phenomenon may be explained by unknown genomic loci acting in *cis* to control methylation patterns [149] or the incomplete erasure of methylation in the germ line [150].

6.1 - Methyltransferase Polymorphisms and Cancer Risk

Genetic disruption of the methyltransferase genes results in altered methylation patterns. Large scale changes such as homozygous deletions are lethal during

mammalian development [151], and small alterations such as single nucleotide polymorphisms (SNPs) are associated with altered methyltransferase expression, particularly that of *DNMT3B* [152-154]. In these instances, over expression of methyltransferase genes are thought to lead to hypermethylation of crucial genes. Mutations within the *DNMT3B* gene are associated with the Immuno-deficiency, Centromere instability and Facial anomalies (ICF) syndrome, a condition which requires two mutated alleles of *DNMT3B*. In addition to the main disease characteristics, abnormal methylation patterns and chromatin defects also result [155]. ICF cells lacking functional DNMT3B show altered expression of genes related to the ICF phenotype, and many of the up-regulated genes show hypomethylation [156]. This is accompanied by histone modifications such as loss of H3K27me3 and gains of the active H3K9 acetylation and H3K4me3 marks. There appear to be no records of cancer in ICF patients, nor in their parents who carry one mutant allele of *DNMT3B*, which suggest mono- and bi-allelic mutations of *DNMT3B* are not a risk factor for cancer development. It is unknown whether certain changes, such as missense mutations, that have yet to be identified may alter the behaviour of the methyltransferase enzymes leading to aberrant DNA methylation patterns that initiate tumour growth.

A report in 2006 by Jones *et al.* [157] found an association between a polymorphism in the methyltransferase gene *DNMT3B* with the age of onset of HNPCC. This polymorphism may represent a genetic mechanism acting in *cis* to regulate DNA methylation. The study in Chapter 2 describes this single nucleotide polymorphism (SNP) in the *DNMT3B* gene and its association with the age of disease onset of HNPCC within an Australian and Polish cohort of individuals at risk of developing disease.

HNPCC is a disease which not only affects the colon but also other epithelial sites such as endometrium, stomach, kidney and ovary [158]. It is not understood which factors are involved in the development of the different disease variants. Similarly the age of onset varies considerably and is thought to be related to both genetic and environmental influences [159]. Previous studies have correlated SNPs with age of disease onset in HNPCC with varying levels of success [159, 160]. Reports of *DNMT3B* polymorphism influencing the age of disease onset in HNPCC patients were of great interest considering *MLH1*, a major gene involved with HNPCC, frequently succumbs to *de novo* DNA methylation in colorectal tumours.

This follow-up study was undertaken to assess the claims from a report in 2006 that described an association between an earlier age of disease onset in HNPCC patients who carried the variant [157]. Confirmation of this result was crucial in determining whether subtle differences in the regulatory region of Δ*DNMT3B* alter gene activity and consequently how this may influence the likelihood of developing disease in a defined population at special risk of malignancy. A confirmatory study was undertaken which failed to substantiate the association of the C>T SNP and earlier age of disease onset in HNPCC patients (Chapter 2). The population size of this study was greater than twice that of the previous study and included participants from both Australia and Poland.

Analysis took into consideration potential population stratification (Australian v Polish) with respect to minor allele frequency and whether or not the genotypes were in Hardy-Weinberg Equilibrium, and the HNPCC mutation type (*MLH1* v

MSH2). Kaplan Meier survival analysis was undertaken and Log-rank, Wilcoxin, Taron-Ware and Cox proportional hazard regression models where employed to test for statistical significance. No significant correlation was observed between any genotype with early onset disease. Similarly no difference was identified when grouping the patients according to their DNA mismatch repair gene mutation and *ΔDNMT3B* SNP genotype (homozygous wild type, heterozygous, homozygous mutant). The sample size of this study was almost three times larger than the previous study by Jones *et al.*, and included 194 cases of confirmed colorectal cancer patients in comparison to the previous 74 cases. Data generated on this population size confer greater statistical confidence and avoid Type 1 or Type 2 errors. Type 1 errors involve 'false positives' whereby an effect is observed when in reality, there is no effect. The conflicting conclusions between this study and that of Jones *et al*. are therefore likely to have been the result of a Type 1 statistical error due to the smaller sample size.

6.1.1 - *DNMT3B* expression

Despite this result, it does not dismiss the possibility that increased expression of the DNMT3B variants is responsible for hypermethylation of key genes, which is also referred to as the hypermethylator phenotype. Numerous studies illustrate that over-expression of the methyltransferase genes is associated with the hypermethylation of key genes, but there is also evidence to suggest this is not always the case. A study by Roll and colleagues in 2008 [161] makes a good argument for the role of DNMT3B over-expression in the hypermethylator phenotype in breast cancer cell lines. Cell lines which displayed

hypermethylation of a group of genes frequently silenced in breast cancer had an elevated level of DNMT3B activity whilst those cell lines in which the genes were not methylated displayed normal levels of DNMT3B. This analysis was centrally focused towards a subset of genes that are altered in the cell lines exhibiting high *DNMT3B* expression, which is an intriguing observation, but the authors may have neglected to find other subsets of genes which were hypermethylated in the cell lines deemed to be low expressors of *DNMT3B* or normalise the expression of methyltransferases against proliferation dependent genes. In contrast, Eads *et al*. [86] showed that a group of genes frequently methylated in colorectal tumours was associated with an increase in expression of all methyltransferases when normalised with β-*actin* or an RNA polymerase subunit, but not with proliferation-dependent genes Histone H4 or PCNA.

The contribution of SNPs within the promoter region to the expression of methyltransferases is a valid mechanism by which differences in cancer risk may occur, yet it remains to be conclusively shown that this mechanism of disease initiation or progression is associated with early onset disease or hypermethylation of specific genes. The debate regarding the over-expression of methyltransferases and hypermethylation of a subset of genes is difficult to conclusively prove and will continue to be a subject of debate.

With the *DNMT3B* SNP failing to show any association with the age of disease onset, the question arose as to whether it was the action of the DNA methyltransferases, and irregularities in this process that could account for the differences in the age of disease onset in HNPCC. Abnormal regulation of

methylation may be controlled by unknown genetic components, however the focus of the next stage of the study was to identify why certain sequences (and associated genes) were targeted for repression in some cell lines but not other cell lines. DNA hypermethylation of crucial genes is a common feature of tumour cells and understanding why repressive modifications are attracted to certain loci remains unknown. As methylation can be erased with methyltransferase inhibitors, an investigation was undertaken to examine how and if global and gene associated CpG island methylation patterns were re-established in cell lines after 5-aza-2'-deoxycytidine treatment was removed.

6.2 - Remethylation response to 5-aza-dC

One of the early observations concerning epigenetic aberrations in tumour cells was the decreased content of genomic DNA methylation in diseased tissue when compared with healthy adjacent tissue from the same patient [162]. The goal of this stage of the study was to analyse methylation levels, and how they respond to treatment with the de-methylating agent 5-aza-dC as described in Chapter 3. The results confirmed that there was a decreased genomic DNA methylation content in untreated cancer cell lines compared to a fibroblast cell line representative of healthy epithelial cells. Treatment with 5-aza-dC resulted in decreased genomic methylation. A decrease of over 50% was observed in all cancer cell lines except HT29, whereas a modest decrease of 25% was observed in the fibroblast cell line. A smaller level of demethylation may indicate a slower rate of growth of some cell lines. 5-aza-dC is an analog of cytosine which needs to be incorporated into newly synthesised DNA strands to exert its effect. As fibroblast cells replicate more slowly than the cancer cells, it is likely the rate of

uptake of 5-aza-dC was slower in these cells, and to some extent the HT29 cells, resulting in lower rates of demethylation.

A study by Stresemann examined the global and gene specific remethylation in myeloid leukaemia cell lines and both bone marrow aspirates and peripheral blood mononuclear cells (PBMCs) from myelodysplastic syndrome (MDS) patients undergoing therapy with methyl-transferase inhibitors [163]. Genomic DNA methylation in the cell lines was reduced by over 1.5% with 5-aza-dC treatment, which was less than that observed in the experiments undertaken herein, but may be explained by the different concentration of inhibitor used. Treatment cycles for patients with MDS lasted four weeks and consisted of 75mg/m^2/day for the first week, with no treatment in the remaining three weeks. Six of the ten MDS patients in the study showed stable disease or positive haematologic responses to the treatment. DNA methylation levels in patients that showed demethylation of PBMCs were restored to baseline levels by the end of each respective treatment cycle. This result is in concordance with the data for the colorectal cancer cell lines and supports the notion that DNA methylation will return to CpG sites from where it was originally removed. As mentioned earlier, this may be due to the re-establishment of DNA methylation on hemi-methylated substrates. Interestingly, the methylation of bone marrow aspirates in one patient remained lower than pretreatment, but this demethylation and that of the PBMCs did not seem to be necessary for a stable or favourable clinical outcome. The results of the above mentioned study and the experiments detailed in this thesis indicate that restoration of genomic methylation levels is a common

outcome following 5-aza-dC exposure, and the reason for effective treatment by this method relies on the reactivation and expression of crucial genes.

There has been little assessment of the remethylation of genomic DNA both *in vitro* and *in vivo.* The return of methylation levels to approximately pre-treatment levels ten days post 5-aza-dC treatment posed an important question. Were the same genes expressed/silenced ten days post treatment or was an altered epigenetic expression pattern established? Upon cessation of 5-aza-dC, a return of global methylation was observed, which almost reached pre-treatment levels in most cell lines assayed. This signifies that these cells lines have not lost their capacity to methylate DNA, and the return of genomic methylation supports the notion that particular genes are deliberately targeted or even marked for repression. However, if the methylation was not completely erased from a given sequence, it may indicate a re-establishment of symmetrical DNA methylation on hemi-methylated DNA rather than specific targeting for inactivation. Surrounding chromatin modifications are also likely to play a role in reactivation or re-silencing and will be discussed shortly.

6.3 - Influence of histone acetylation on remethylation

The synergistic reactivation of genes with the demethylation agent 5-aza-dC and the histone deacetylase inhibitor TSA have been well documented [60, 147, 148]. An examination was undertaken as to whether a constant exposure of TSA to the colorectal cancer cells would alter DNA demethylation or the process of remethylation. A correlation between the two modifications may potentially be exploited to reactivate greater numbers of genes, enhancing the possibility of

effective drug treatment. In the study described in chapter 3, two groups of HCT116 cells were treated with 5-aza-dC and one group was also treated with TSA at 100nM to induce widespread hyperacetylation. After 72 hours 5-aza-dC was removed from both groups, and TSA exposure was maintained for the following 10 days. No difference in demethylation was detected in cells co-treated with TSA, nor was the remethylation process altered. Taken together with evidence from previous studies, the combined treatments may synergistically reactivate genes, but acetylated histones do not appear to affect the remethylation of CpG sites, or to be more specific, acetylated histones do not appear to deter DNA methyltransferase enzymes from unmethylated substrates.

6.4 - Specific responses of CpG island methylation to 5-aza-dC

An aim of this aspect of the study was to examine how DNA methylation patterns were re-established after they had been erased with 5-aza-dC. Would an aberrantly hypermethylated gene remain expressed when the drug was removed? Global methylation results suggested a decrease of approximately 50% was to be expected. In cells treated with 5-aza-dC, the CpG islands of the genes assessed underwent demethylation of up to ~30% at single CpG sites as determined by direct bisulfite sequencing. The majority of CpG sites assayed however only revealed minor demethylation in comparison with the demethylation observed at a global level, and the gene-specific pattern of methylation remained largely unchanged. Although the expression may be driven from partially demethylated alleles following 5-aza-dC, transcription and active gene expression was detected from genes at ten days post treatment where methylation at CpG sites was almost identical to that of untreated cells. For these genes it is possible that certain

epigenetic features are involved with maintaining expression, and will be discussed shortly.

The discrepancy between gene-associated CpG islands and global DNA methylation levels is likely to be explained by the demethylation of repetitive elements, Alu repeats, transposable elements, satellite and non-satellite sequences, which make up a large proportion of the genome [77, 164-166]. Similar results were identified by Lim *et al.* [167] who found that methylation in genic regions decreased only at certain concentrations of 5-aza-dC and that repetitive elements were predominantly affected.

Numerous studies have used 5-aza-dC in the reactivation of specific genes and demonstrated that demethylation occurs, however the most common methodology for assessing this is methylation sensitive PCR. While this is a sensitive method for detecting unmethylated template, it is not quantitative and small changes in methylation have been disproportionately reported following exponential PCR amplification. Direct sequencing was chosen for this study due to cost effectiveness when studying a number of genes, and as a means to quantitatively assess DNA methylation and changes in methylation across CpG islands.

6.5 - Localised hypomethylation at Transcription Start Sites

An important factor that is frequently overlooked is the resulting pattern of gene expression post 5-aza-dC treatment. Experiments detailed in Chapter 3 identified two classes of genes; short and long term reactivated genes. A reasonable

presumption would be that long term reactivated genes undergo DNA demethylation and remain hypomethylated. Bisulfite sequencing revealed this was not the case and minimal change to the methylation landscape was detected at the CpG islands examined. The genes with long term reactivation displayed a unique pattern of DNA methylation at the transcription start sites, namely localised hypomethylation surrounded by hypermethylation. Following 5-aza-dC treatment, this localised hypomethylation is presumed to initiate an altered histone modification profile that allows prolonged transcription. The histone modifications at these genes were examined by chromatin immunoprecipitation and are discussed shortly.

DNA methylation of a CpG island is commonly accepted to be repressive of transcriptional machinery. However the effect of methylation is not always uniform and determining if genes are expressed in instances of epigenetic change is difficult and is likely to depend on other epigenetic features. In the work described in Chapter 3, bisulfite sequencing revealed common and unique patterns of DNA methylation at individual CpG islands. Generally patterns of DNA methylation correlate with expression in untreated cells, i.e. hypomethylation was observed in expressed genes and no expression was detected from hypermethylated genes. The identification of unique CpG island methylation included genes which were expressed at very low levels such as *ZFP3* in SW480 cells and genes which are likely to be expressed exclusively from one allele, such as *CDKN2A* in HCT116 cells.

These results lead to the identification of the importance of DNA hypomethylation at the transcriptional start site (TSS). Several genes displayed localised hypomethylated cytosines adjacent to the TSS and were surrounded by numerous hypermethylated CpG sites elsewhere in that CpG island. Whilst it is known that promoter CpG methylation affects transcriptional activity and methylation elsewhere in the gene has little or no effect, this result may suggest the region in which DNA methylation can regulate transcription may be as small as 100bp or less. It has been shown that MeCP2 is capable of binding to a single methylated CpG site, so it seems reasonable to suggest that a small region may be void of repressive proteins even if they are located only a short distance away from a CpG island.

An intriguing question resulting from this data concerns how such a DNA methylation pattern is established. A possible explanation may involve the mechanism of action of the DNA methyltransferases. Previous studies have examined the domains of the DNA methyltransferases with respect to the genomic region they target. Studies on the catalytic domains of the methyltransferases examining the establishment of methylation patterns have revealed the DNMT1 and DNMT3B are processive whilst the DNMT3A protein acts in a distributive manner [168]. For a small group of CpG sites to remain hypomethylated, or even resistant to methylation, establishment of this pattern may involve methylation of surrounding CpG sites in a distributive manner by *de novo* methyltransferases. A processive *de novo* methyltransferase would result in uniform methylation, and the pattern would subsequently be faithfully retained by the processive DNMT1 during cellular replication. The pattern of methylation

may have originated during early development when the gene was being expressed and widespread genomic methylation was occurring. Transcription factors or other proteins may have been bound to the DNA at the hypomethylated sites and eventually the gene was no longer expressed but the methylation and chromatin had already been 'set' for that gene. The pattern of DNA methylation at that site may merely be an artifact of past events. Another explanation may involve the positioning of DNA and nucleosomes. Nucleosomal bound DNA has been shown to be more highly methylated than flanking DNA and linker regions of DNA are approximately 30bp [169]. This 30bp region either intentionally or coincidently may correspond with the transcriptional start sites resulting in the unique methylation patterns observed in some genes.

6.6 - Expression Array analysis of gene expression

In the treatment of MDS it has been demonstrated that patients who respond positively to treatment from demethylating agents display altered DNA methylation levels in both bone marrow aspirates and peripheral blood mononuclear cells [163]. Furthermore, the methylation levels were restored to baseline changes between each treatment cycle. However, this de- and remethylation induces a different pattern of gene expression. Experiments detailed in Chapter 5 detail the effects of 5-aza-dC and TSA on gene expression in colorectal cancer cells. This study served to identify patterns of gene expression during and after 5-aza-dC exposure to examine which pathways were up and down-regulated. This information may prove useful in determining the success of 5-aza-dC or why this treatment is ineffective in the treatment of some tumours.

Exposure to 5-aza-dC induced the expression of over 1000 genes in each cell line examined, and just over half of these were associated with a CpG island. Whilst it is difficult to determine what fraction of these were non-specific or due to secondary epigenetic events (alteration of histone proteins) it demonstrates the ability of 5-aza-dC to affect non-CpG associated genes, which lead to non-specific effects. Concerns have been raised regarding the off-target effects of 5-aza-dC on normally methylated regions of DNA [170] and non-specific activation of oncogenic pathways [171]. The success of 5-aza-dC may depend on activation or repression of as-yet unidentified off-target pathways in some cell types.

A major hurdle encountered with the use of demethylation agents against disease is remethylation of reactivated gene promoters upon cessation of treatment [172, 173]. Critical genes that become reactivated revert to an inactive state without lasting effect. Numerous strategies could be employed to combat this, including sustained treatment with demethylating drugs [174, 175] or combined treatments with Histone Deacetylase Inhibitors [60, 176-178]. Our results indicate a combined treatment with 5-aza-dC followed with sustained TSA treatment may counteract re-suppression by altering DNA methylation and histone modifications to allow prolonged gene reactivation. Ultimately, the combinations or time course necessary for successful treatment may depend on tissue type.

Recently, epigenetic modifications have been altered with 5-aza-dC and TSA such that a reprogramming of the cell occurs. In attempting to clone bovine embryos, Ding *et al*. (2008) were able to increase the formation of blastocysts

and total cell numbers by treating donor cells and cloned embryos with 5-aza-dC and TSA [179]. This was aimed to eliminate the issue of any pre-existing programming from the donor DNA, and may be necessary to mimic the wave of DNA demethylation that occurs shortly after conception [179]. Similarly, there are reports of reprogramming and de-differentiation of cells treated with 5-aza-dC and TSA [180, 181]. Interested in generating pluripotent stem cells for the treatment of diseases such as diabetes and Parkinson's disease, Zhang *et al.* were able to dedifferentiate fibroblasts with 5-aza-dC and TSA. This was achieved with the reactivation of transcription factors that are expressed in pluripotent embryonic stem cells. We sought to examine how closely the pattern of gene expression in treated colorectal cancer cell lines could be altered to resemble that of healthy epithelial cells (Chapter 5). On a genome wide level, gene expression profiles within cell lines remain similar following treatment as shown by the unsupervised clustering of all genes and cell lines, regardless of 5-aza-dC treatment time point. This indicates that expression profiles are not readily manipulated and a resetting effect does not occur in the colorectal cancer cells such that expression resembles that of healthy epithelial cells.

Pathway analysis of up and down-regulated genes following treatment found that expected gene ontology families such as the apoptotic pathways were not significantly affected. Instead, several pathways such as Calcium signalling pathway and neuroactive ligand-receptor interaction genes were up-regulated. In terms of commonly down-regulated genes, the neuroactive ligand-receptor interaction, olfactory transduction and Asthma pathways were commonly affected. The neuroactive ligand-receptor family of genes was also significantly

overrepresented in the up-regulated gene list, indicative of secondary effects to compensate for changes induced by drug treatment. These pathways may also be indicative of further non-specific effects of 5-aza-dC.

Although the pathway was not significantly altered, there were individual genes from the Apoptosis pathway which were altered after 5-aza-dC exposure. The PRKACB protein is involved with the phosphorylation of BCL2, an agonist of cell death, which ultimately leads to cell survival. At least one variant of the *PRKACB* gene was not expressed in fibroblast cells and is down-regulated to non-detectable levels in the HT29, SW48 and LoVo cells following 5-aza-dC treatment. This mechanism may normally induce apoptosis, yet appears insufficient to have the same affect in these cell lines. This may be explained by the different behavior of immortalised cell lines *in vitro* compared with the behavior *in vivo*. Predominantly, the pathways which were altered with 5-aza-dC exposure in colorectal cancer cell lines do not appear related to malignancy and are likely to explain why this treatment is not an effective treatment for tumours of this nature. In haematologic malignancies where 5-aza-dC is an effective treatment, the drug may induce apoptosis or cell cycle pathways in the bone marrow stem cells which are the origin of the disease.

The genes selected for ChIP analysis were chosen based on their responsiveness to 5-aza-dC treatment and resulting gene expression, or more specifically, the variation in expression following removal of 5-aza-dC, which suggested the epigenetic status of these genes was re-established differently. Of particular interest to this study are the genes which possessed hypomethylated cytosines, as

they did not show large DNA demethylation and remained expressed 10 days after 5-aza-dC treatment. These genes became the centre of our investigation into the change in chromatin status after drug treatment.

6.7 - Changes to Histone Acetylation and methylation

Much attention has been paid to the mode of action of 5-aza-dC and its reactivating ability; however an important facet of this treatment is likely to revolve around the resulting pattern of expression when the treatment period is finished. The studies in chapter 3 and 4 identified reactivated genes that remained expressed or reverted to an inactive state. The discordance between the gene-specific DNA methylation levels and expression levels of the genes examined in Chapter 4 indicated transcription of these genes may be influenced by histone modifications. There was also evidence that hypomethylated cytosine in the immediate vicinity of the transcription start site was involved. Therefore an investigation of chromatin modification was undertaken in Chapter 4. This demonstrated the involvement of histone factors in long term re-expression and would also aid with understanding the mechanisms of remethylation, repression or prolonged transcription.

In the study in Chapter 4, four common histone modifications were assessed regarding their role in the long term reactivation of expression. It became apparent that certain generalisations regarding these modifications could be made depending on the resulting level of transcription.

Genes that were constitutively expressed displayed strong association with active modification such as H3Ac and H3K4me3 and with treatment these were stable or showed minor increases. Genes that became up-regulated after drug treatment (such as *ZFP3* in SW480 cells) displayed DNA methylation that was varied, but usually involved hypomethylation surrounding the transcription start site. In terms of chromatin, these genes displayed increases in H3Ac, H3K4me3 and also H3K9me3 which is typically associated with inactive chromatin. Ten days after 5-aza-dC was removed, an increase in H3K4me3 above untreated levels was still detected. The difference between short and long term reactivated genes was more profound. Upon 5-aza-dC treatment, H3Ac became increased in long term reactivated genes, but not in short term expressed genes. After 10 days of drug free growth, a reduction of H3K27me3 (when originally present) in unison with a strong increase of H3Ac were introduced at the transcription start site of long term reactivated genes. The increase in H3Ac was not increased at any time point in the short term expressed genes.

The acetylation of histone H3 is evidently a solid activator of gene expression, and was a stronger indicator of gene activity than the other modifications assessed. When these results were taken into consideration with the DNA methylation profile across the transcription start site, it began to become apparent how methylation and chromatin interact to control expression; recruitment of histone H3 acetylation appeared to be directly related to localised cytosine hypomethylation at the transcriptional start site. It is not clear why such a methylation pattern would exist as genes of varying biological function were affected, and it may be an artifact of previous methylation patterns, as discussed

earlier. Localised hypomethylation has been described previously in the Oncostatin M gene [182], but the role of this methylation pattern on long term reactivation was not investigated on this occasion. Dependent on the sequence, unmethylated CpGs may create a stretch of hypomethylated DNA ranging in size from 20bp (as seen in the CDO1 gene) up to 50bp (as seen in the *HSPC105* or *MAGEA3* genes) or larger. This is sufficient to allow recruitment of H3Ac and permit transcription, despite neighboring CpG hypermethylation.

6.8 - Overall conclusions

In conclusion, the studies described in this thesis have discussed several interesting mechanisms regarding the epigenetic control of gene expression.

i) An investigation on the polymorphism within the *DNMT3B* gene was found to have no effect on the age of disease onset in HNPCC patients, disagreeing with previous claims. The idea at the centre of this study involves a potential mechanism by which a genetic event may regulate the epigenetic status of a gene at a separate locus, but the concept was not substantiated in this instance.

ii) The methylation of genomic DNA is an important factor in tumour cells, however the changes to genomic levels do not equate to changes in gene specific levels, denoting that not all regions of the genome are affected equally by 5-aza-dC.

iii) Alteration to the expression level of epigenetically silenced genes appears related to the pre-existing DNA methylation level, or more specifically the localised methylation at the transcription start sites.

iv) Analysis of genome wide expression levels revealed that apoptotic and cell control pathways were not activated. Some genes involved with apoptosis were affected, but do not appear sufficient to activate the pathway in these cells.

v) The accumulation of acetylated histone at hypomethylated transcription start sites following 5-aza-dC treatment appears to be necessary for long term transcriptional reactivation. Without localised NA hypomethylation, acetylation of histone H3 was not increased, and without either of these modifications no long term change of expression was detected.

6.9 - Future Directions

From the studies described in this thesis, there are several avenues that could be followed up to reveal further information on the epigenetic regulation of the genome.

There appears to be cooperation of DNA hypomethylation and histone acetylation to reverse transcriptional silencing. Determination of the mechanism that facilitates the recruitment of histone acetyltransferase to hypomethylated DNA will generate valuable information that may be used to understand the reactivation or repression of epigenetically silenced genes. Utilizing cell lines of other tumour types to further determine whether the identified methylation patterns are present in these tumours would make this work directly applicable to other forms of cancer. The use of a xenograft model may be an excellent way to examine the dynamic of epigenetic regulation of these genes *in vivo*.

Another aspect to investigate would be the identification of large intervening non-coding RNAs (lincRNAs), such as HOTAIR. A recent study has shown that its expression is capable of altering chromatin modifications which lead increased invasiveness of tumour cells and interestingly, its expression is closely associated with patient prognosis [183]. Other non-coding RNA species may also be of interest in the activation or repression of distal genes.

The non-specific action of 5-aza-dC may have adverse affects on the genome that are yet to be comprehensively described. Demethylation of proto-oncogenes may exacerbate tumour cell growth, overriding any beneficial effect of the treatment. Similarly, reactivation of silenced genes such as *MLH1* will be detrimental if the genome of that cell has incurred lesions as a result of suppressed MLH1 function. Also, chromosomal instability often exhibited in tumour cells may be further worsened with 5-aza-dC, although this may provoke a favourable apoptotic response.

In terms of treating disease with 5-aza-dC, it appears that a cluster of disease/tissue specific genes must be reactivated for a positive outcome driven by either a resetting of expression patterns or induction of cell death. Whether they are directly activated via demethylation or are up-regulated as a secondary effect, identification of these genes would be a valuable asset. When the genes are known, the suitability of 5-aza-dC treatment could be assessed by examining the methylation profiles at these target genes. Without specific hypomethylation, other methods may need to be employed to re-express the silenced transcripts.

There are now several classes of methyltransferase inhibitors available, including zebularine, 5-azacytidine and its analogs 5,6-dihydro-5-azacytidine, α-5-aza-2'-deoxycytidine. In a recent study it was shown that 2'-deoxycytidine-5,6-dihydro-5-azacytidine was less cytotoxic and more stable than 5-aza-2'-dC at doses which elicit comparable hypomethylation and gene reactivation [184]. There are also new classes of histone deacetylase inhibitors such as suberoylanilide hydroxamic acid (SAHA) that may confer synergistic effects when used with methyltransferase inhibitors [185] that are absent with long known agents such as TSA.

Gene expression profiling of leukaemic cells representative of tissue that is effectively treated with 5-aza-dC can reveal differences in gene expression pathways between a responsive and non-responsive treatment. Knowledge of the molecular events which are necessary for positive treatment outcome may be used as a predictive tool to distinguish between individuals that respond to 5-aza-dC treatment.

The identification of the proteins responsible for active demethylation of DNA remain to be one of the biggest mysteries in the field of epigenetics. Active removal of methyl groups is understood to occur shortly after conception but the components involved with this process remain elusive. There is great potential for manipulation of the demethylation process in understanding, treatment and possibly prevention of disease.

6.10 - Summary

These observations described in this thesis have identified interesting mechanisms of epigenetic control of gene expression. They further define the current understanding of epigenetic gene regulation and have identified factors that need to be overcome in the treatment of epigenetic aberrations in both colorectal cancer and other forms of malignancy. In addition, this work also contributes to the understanding of the broader subject of gene expression.

Chapter 7 - Bibliography

1. Stein L D. (2004) Human genome: end of the beginning. Nature; 431(7011): p. 915-6.
2. Wheeler H L and Johnson T B. (1903) Researches on Pyrimidine Derivatives: 5-Methyl-cytosine. Am. Chem. J.; 31: p. 591-606.
3. Johnson T B and Coghill R D. (1925) The Discovery Of 5-Methyl-Cytosine In Tuberculinic Acid, The Nucleic Acid Of Tubercle Bacillus. J. Am. Chem. Soc; 47(11): p. 2838-2844.
4. Hotchkiss R D. (1948) The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. J Biol Chem; 175(1): p. 315-32.
5. Holliday R and Pugh J E. (1975) DNA modification mechanisms and gene activity during development. Science; 187(4173): p. 226-32.
6. Dupont C, Armant D R, and Brenner C A. (2009) Epigenetics: definition, mechanisms and clinical perspective. Semin Reprod Med; 27(5): p. 351-7.
7. Bergmann A and Lane M E. (2003) HIDden targets of microRNAs for growth control. Trends Biochem Sci; 28(9): p. 461-3.
8. Lister R, Pelizzola M, Dowen R H, Hawkins R D, Hon G, et al. (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature; 462(7271): p. 315-22.
9. Berletch J B, Phipps S M, Walthall S L, Andrews L G, and Tollefsbol T O. (2007) A method to study the expression of DNA methyltransferases in aging systems in vitro. Methods Mol Biol; 371: p. 81-7.
10. Yoder J A, Soman N S, Verdine G L, and Bestor T H. (1997) DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. J Mol Biol; 270(3): p. 385-95.
11. Leonhardt H, Page A W, Weier H U, and Bestor T H. (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell; 71(5): p. 865-73.
12. Bestor T H. (2000) The DNA methyltransferases of mammals. Hum Mol Genet; 9(16): p. 2395-402.
13. Rhee I, Bachman K E, Park B H, Jair K W, Yen R W, et al. (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature; 416(6880): p. 552-6.
14. Fatemi M, Hermann A, Gowher H, and Jeltsch A. (2002) Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. Eur J Biochem; 269(20): p. 4981-4.
15. Holz-Schietinger C and Reich N O. (2010) The inherent processivity of the human de novo methyltransferase 3A (DNMT3A) is enhanced by DNMT3L. J Biol Chem; 285(38): p. 29091-100.
16. Wienholz B L, Kareta M S, Moarefi A H, Gordon C A, Ginno P A, et al. (2010) DNMT3L modulates significant and distinct flanking sequence preference for DNA methylation by DNMT3A and DNMT3B in vivo. PLoS Genet; 6(9).

17. Hata K, Okano M, Lei H, and Li E. (2002) Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development; 129(8): p. 1983-93.
18. Chedin F, Lieber M R, and Hsieh C L. (2002) The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. Proc Natl Acad Sci U S A; 99(26): p. 16916-21.
19. Suetake I, Shinozaki F, Miyagawa J, Takeshima H, and Tajima S. (2004) DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. J Biol Chem; 279(26): p. 27816-23.
20. Hermann A, Schmitt S, and Jeltsch A. (2003) The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. J Biol Chem; 278(34): p. 31717-21.
21. Goll M G, Kirpekar F, Maggert K A, Yoder J A, Hsieh C L, et al. (2006) Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science; 311(5759): p. 395-8.
22. Jeanpierre M, Turleau C, Aurias A, Prieur M, Ledeist F, et al. (1993) An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet; 2(6): p. 731-5.
23. Miniou P, Jeanpierre M, Blanquet V, Sibella V, Bonneau D, et al. (1994) Abnormal methylation pattern in constitutive and facultative (X inactive chromosome) heterochromatin of ICF patients. Hum Mol Genet; 3(12): p. 2093-102.
24. Kanai Y, Ushijima S, Nakanishi Y, Sakamoto M, and Hirohashi S. (2003) Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. Cancer Lett; 192(1): p. 75-82.
25. Bartolomei M S and Ferguson-Smith A C. (2011) Mammalian Genomic Imprinting. Cold Spring Harb Perspect Biol; (16).
26. DeChiara T M, Robertson E J, and Efstratiadis A. (1991) Parental imprinting of the mouse insulin-like growth factor II gene. Cell; 64(4): p. 849-59.
27. Vambergue A, Fajardy I, Dufour P, Valat A S, Vandersippe M, et al. (2007) No loss of genomic imprinting of IGF-II and H19 in placentas of diabetic pregnancies with fetal macrosomia. Growth Horm IGF Res; 17(2): p. 130-6.
28. Leighton P A, Saam J R, Ingram R S, Stewart C L, and Tilghman S M. (1995) An enhancer deletion affects both H19 and Igf2 expression. Genes Dev; 9(17): p. 2079-89.
29. Bell A C and Felsenfeld G. (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature; 405(6785): p. 482-5.
30. Elkin M, Shevelev A, Schulze E, Tykocinsky M, Cooper M, et al. (1995) The expression of the imprinted H19 and IGF-2 genes in human bladder carcinoma. FEBS Lett; 374(1): p. 57-61.
31. Matouk I J, DeGroot N, Mezan S, Ayesh S, Abu-lail R, et al. (2007) The H19 non-coding RNA is essential for human tumor growth. PLoS One; 2(9): p. e845.
32. Brown C J, Ballabio A, Rupert J L, Lafreniere R G, Grompe M, et al. (1991) A gene from the region of the human X inactivation centre is

expressed exclusively from the inactive X chromosome. Nature; 349(6304): p. 38-44.
33. Okamoto I, Otte A P, Allis C D, Reinberg D, and Heard E. (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. Science; 303(5658): p. 644-9.
34. Wutz A and Gribnau J. (2007) X inactivation Xplained. Curr Opin Genet Dev; 17(5): p. 387-93.
35. Vogelstein B, Fearon E R, Hamilton S R, Preisinger A C, Willard H F, et al. (1987) Clonal analysis using recombinant DNA probes from the X-chromosome. Cancer Res; 47(18): p. 4806-13.
36. Pegoraro E, Whitaker J, Mowery-Rushton P, Surti U, Lanasa M, et al. (1997) Familial skewed X inactivation: a molecular trait associated with high spontaneous-abortion rate maps to Xq28. Am J Hum Genet; 61(1): p. 160-70.
37. Plenge R M, Stevenson R A, Lubs H A, Schwartz C E, and Willard H F. (2002) Skewed X-chromosome inactivation is a common feature of X-linked mental retardation disorders. Am J Hum Genet; 71(1): p. 168-73.
38. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, et al., *Molecular Biology of The Cell*. 4th ed, ed. S. Gibbs. 2002, New York: Garland Science.
39. Kornberg R D. (1977) Structure of chromatin. Annu Rev Biochem; 46: p. 931-54.
40. Vignali M and Workman J L. (1998) Location and function of linker histones. Nat Struct Biol; 5(12): p. 1025-8.
41. Bednar J, Horowitz R A, Grigoryev S A, Carruthers L M, Hansen J C, et al. (1998) Nucleosomes, linker DNA, and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin. Proc Natl Acad Sci U S A; 95(24): p. 14173-8.
42. Owen-Hughes T and Workman J L. (1994) Experimental analysis of chromatin function in transcription control. Crit Rev Eukaryot Gene Expr; 4(4): p. 403-41.
43. Juan L J, Utley R T, Adams C C, Vettese-Dadey M, and Workman J L. (1994) Differential repression of transcription factor binding by histone H1 is regulated by the core histone amino termini. Embo J; 13(24): p. 6031-40.
44. Khorasanizadeh S. (2004) The nucleosome: from genomic organization to genomic regulation. Cell; 116(2): p. 259-72.
45. Herman J G and Baylin S B. (2003) Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med; 349(21): p. 2042-54.
46. Baylin S B. (2005) DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol; 2 Suppl 1(1): p. S4-11.
47. Meehan R R, Lewis J D, McKay S, Kleiner E L, and Bird A P. (1989) Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell; 58(3): p. 499-507.
48. Boyes J and Bird A. (1991) DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell; 64(6): p. 1123-34.

49. Padjen K, Ratnam S, and Storb U. (2005) DNA methylation precedes chromatin modifications under the influence of the strain-specific modifier Ssm1. Mol Cell Biol; 25(11): p. 4782-91.
50. Meehan R R, Lewis J D, and Bird A P. (1992) Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. Nucleic Acids Res; 20(19): p. 5085-92.
51. Brown S E and Szyf M. (2007) Epigenetic programming of the rRNA promoter by MBD3. Mol Cell Biol; 27(13): p. 4938-52.
52. Zhang Y, Ng H H, Erdjument-Bromage H, Tempst P, Bird A, et al. (1999) Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev; 13(15): p. 1924-35.
53. Ballestar E and Wolffe A P. (2001) Methyl-CpG-binding proteins. Targeting specific gene repression. Eur J Biochem; 268(1): p. 1-6.
54. Shahbazian M D and Grunstein M. (2007) Functions of site-specific histone acetylation and deacetylation. Annu Rev Biochem; 76: p. 75-100.
55. van Holde K, *Chromatin,* ed. A. Rich. 1988, New York: Springer-Verlag.
56. Kornberg R D and Lorch Y. (1999) Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell; 98(3): p. 285-94.
57. McGhee J D and Felsenfeld G. (1980) Nucleosome structure. Annu Rev Biochem; 49: p. 1115-56.
58. Lowell J E and Pillus L. (1998) Telomere tales: chromatin, telomerase and telomere function in Saccharomyces cerevisiae. Cell Mol Life Sci; 54(1): p. 32-49.
59. Lee D Y, Hayes J J, Pruss D, and Wolffe A P. (1993) A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell; 72(1): p. 73-84.
60. Cameron E E, Bachman K E, Myohanen S, Herman J G, and Baylin S B. (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet; 21(1): p. 103-7.
61. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, et al. (2002) A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet; 31(2): p. 141-9.
62. Kuo M H and Allis C D. (1998) Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays; 20(8): p. 615-26.
63. Rice J C and Allis C D. (2001) Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Cell Biol; 13(3): p. 263-73.
64. Rea S, Eisenhaber F, O'Carroll D, Strahl B D, Sun Z W, et al. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature; 406(6796): p. 593-9.
65. Shi Y, Lan F, Matson C, Mulligan P, Whetstine J R, et al. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell; 119(7): p. 941-53.
66. Sims R J, 3rd, Nishioka K, and Reinberg D. (2003) Histone lysine methylation: a signature for chromatin function. Trends Genet; 19(11): p. 629-39.

67. Santos-Rosa H, Schneider R, Bannister A J, Sherriff J, Bernstein B E, et al. (2002) Active genes are tri-methylated at K4 of histone H3. Nature; 419(6905): p. 407-11.
68. Chew Y C, West J T, Kratzer S J, Ilvarsonn A M, Eissenberg J C, et al. (2008) Biotinylation of histones represses transposable elements in human and mouse cells and cell lines and in Drosophila melanogaster. J Nutr; 138(12): p. 2316-22.
69. Thiriet C and Hayes J J. (2008) Linker histone phosphorylation regulates global timing of replication origin firing. J Biol Chem.
70. Wu J, Huen M S, Lu L Y, Ye L, Dou Y, et al. (2008) Histone ubiquitination associates with BRCA1-Dependent DNA Damage Response. Mol Cell Biol.
71. Shiio Y and Eisenman R N. (2003) Histone sumoylation is associated with transcriptional repression. Proc Natl Acad Sci U S A; 100(23): p. 13225-30.
72. Jubb A M, Bell S M, and Quirke P. (2001) Methylation and colorectal cancer. J Pathol; 195(1): p. 111-34.
73. Feinberg A P and Vogelstein B. (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature; 301(5895): p. 89-92.
74. Holm T M, Jackson-Grusby L, Brambrink T, Yamada Y, Rideout W M, 3rd, et al. (2005) Global loss of imprinting leads to widespread tumorigenesis in adult mice. Cancer Cell; 8(4): p. 275-85.
75. Goelz S E, Vogelstein B, Hamilton S R, and Feinberg A P. (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. Science; 228(4696): p. 187-90.
76. Liu L, Wylie R C, Andrews L G, and Tollefsbol T O. (2003) Aging, cancer and nutrition: the DNA methylation connection. Mech Ageing Dev; 124(10-12): p. 989-98.
77. Lander E S, Linton L M, Birren B, Nusbaum C, Zody M C, et al. (2001) Initial sequencing and analysis of the human genome. Nature; 409(6822): p. 860-921.
78. Matsuzaki K, Deng G, Tanaka H, Kakar S, Miura S, et al. (2005) The relationship between global methylation level, loss of heterozygosity, and microsatellite instability in sporadic colorectal cancer. Clin Cancer Res; 11(24 Pt 1): p. 8564-9.
79. Weisenberger D J, Campan M, Long T I, Kim M, Woods C, et al. (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res; 33(21): p. 6823-36.
80. Furukawa T, Konishi F, Masubuchi S, Shitoh K, Nagai H, et al. (2002) Densely methylated MLH1 promoter correlates with decreased mRNA expression in sporadic colorectal cancers. Genes Chromosomes Cancer; 35(1): p. 1-10.
81. Belinsky S A, Nikula K J, Palmisano W A, Michels R, Saccomanno G, et al. (1998) Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci U S A; 95(20): p. 11891-6.
82. Kanai Y, Ushijima S, Kondo Y, Nakanishi Y, and Hirohashi S. (2001) DNA methyltransferase expression and DNA methylation of CPG islands

and peri-centromeric satellite regions in human colorectal and stomach cancers. Int J Cancer; 91(2): p. 205-12.
83. Jin F, Dowdy S C, Xiong Y, Eberhardt N L, Podratz K C, et al. (2005) Up-regulation of DNA methyltransferase 3B expression in endometrial cancers. Gynecol Oncol; 96(2): p. 531-8.
84. Melki J R, Warnecke P, Vincent P C, and Clark S J. (1998) Increased DNA methyltransferase expression in leukaemia. Leukemia; 12(3): p. 311-6.
85. Belinsky S A, Nikula K J, Baylin S B, and Issa J P. (1996) Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. Proc Natl Acad Sci U S A; 93(9): p. 4045-50.
86. Eads C A, Danenberg K D, Kawakami K, Saltz L B, Danenberg P V, et al. (1999) CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. Cancer Res; 59(10): p. 2302-6.
87. Kimura F, Seifert H H, Florl A R, Santourlidis S, Steinhoff C, et al. (2003) Decrease of DNA methyltransferase 1 expression relative to cell proliferation in transitional cell carcinoma. Int J Cancer; 104(5): p. 568-78.
88. De Marzo A M, Marchi V L, Yang E S, Veeraswamy R, Lin X, et al. (1999) Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. Cancer Res; 59(16): p. 3855-60.
89. Esteller M, Corn P G, Baylin S B, and Herman J G. (2001) A gene hypermethylation profile of human cancer. Cancer Res; 61(8): p. 3225-9.
90. Feltus F A, Lee E K, Costello J F, Plass C, and Vertino P M. (2003) Predicting aberrant CpG island methylation. Proc Natl Acad Sci U S A; 100(21): p. 12253-8.
91. Gjerset R A and Martin D W, Jr. (1982) Presence of a DNA demethylating activity in the nucleus of murine erythroleukemic cells. J Biol Chem; 257(15): p. 8581-3.
92. Weiss A, Keshet I, Razin A, and Cedar H. (1996) DNA demethylation in vitro: involvement of RNA. Cell; 86(5): p. 709-18.
93. Jost J P. (1993) Nuclear extracts of chicken embryos promote an active demethylation of DNA by excision repair of 5-methyldeoxycytidine. Proc Natl Acad Sci U S A; 90(10): p. 4684-8.
94. Swisher J F, Rand E, Cedar H, and Marie Pyle A. (1998) Analysis of putative RNase sensitivity and protease insensitivity of demethylation activity in extracts from rat myoblasts. Nucleic Acids Res; 26(24): p. 5573-80.
95. Cortazar D, Kunz C, Saito Y, Steinacher R, and Schar P. (2007) The enigmatic thymine DNA glycosylase. DNA Repair (Amst); 6(4): p. 489-504.
96. Bhattacharya S K, Ramchandani S, Cervoni N, and Szyf M. (1999) A mammalian protein with specific demethylase activity for mCpG DNA. Nature; 397(6720): p. 579-83.
97. Hamm S, Just G, Lacoste N, Moitessier N, Szyf M, et al. (2008) On the mechanism of demethylation of 5-methylcytosine in DNA. Bioorg Med Chem Lett; 18(3): p. 1046-9.

98. Ng H H, Zhang Y, Hendrich B, Johnson C A, Turner B M, et al. (1999) MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nat Genet; 23(1): p. 58-61.
99. Chatagnon A, Bougel S, Perriaud L, Lachuer J, Benhattar J, et al. (2008) Specific association between the Methyl-CpG binding domain protein 2 and the hypermethylated region of the human telomerase reverse transcriptase promoter in cancer cells. Carcinogenesis.
100. Hendrich B, Guy J, Ramsahoye B, Wilson V A, and Bird A. (2001) Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. Genes Dev; 15(6): p. 710-23.
101. Metivier R, Gallais R, Tiffoche C, Le Peron C, Jurkowska R Z, et al. (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature; 452(7183): p. 45-50.
102. Kangaspeska S, Stride B, Metivier R, Polycarpou-Schwarz M, Ibberson D, et al. (2008) Transient cyclical methylation of promoter DNA. Nature; 452(7183): p. 112-5.
103. Wu J C and Santi D V. (1985) On the mechanism and inhibition of DNA cytosine methyltransferases. Prog Clin Biol Res; 198: p. 119-29.
104. Choi S W, Friso S, Ghandour H, Bagley P J, Selhub J, et al. (2004) Vitamin B-12 deficiency induces anomalies of base substitution and methylation in the DNA of rat colonic epithelium. J Nutr; 134(4): p. 750-5.
105. Takiguchi M, Achanzar W E, Qu W, Li G, and Waalkes M P. (2003) Effects of cadmium on DNA-(Cytosine-5) methyltransferase activity and DNA methylation status during cadmium-induced cellular transformation. Exp Cell Res; 286(2): p. 355-65.
106. Costa M, Davidson T L, Chen H, Ke Q, Zhang P, et al. (2005) Nickel carcinogenesis: epigenetics and hypoxia signaling. Mutat Res; 592(1-2): p. 79-88.
107. Bonsch D, Lenz B, Fiszer R, Frieling H, Kornhuber J, et al. (2006) Lowered DNA methyltransferase (DNMT-3b) mRNA expression is associated with genomic DNA hypermethylation in patients with chronic alcoholism. J Neural Transm; 113(9): p. 1299-304.
108. Tollefsbol T O and Hutchison C A, 3rd. (1997) Control of methylation spreading in synthetic DNA sequences by the murine DNA methyltransferase. J Mol Biol; 269(4): p. 494-504.
109. Knudson A G, Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A; 68(4): p. 820-3.
110. Ohtani-Fujita N, Dryja T P, Rapaport J M, Fujita T, Matsumura S, et al. (1997) Hypermethylation in the retinoblastoma gene is associated with unilateral, sporadic retinoblastoma. Cancer Genet Cytogenet; 98(1): p. 43-9.
111. Prowse A H, Webster A R, Richards F M, Richard S, Olschwang S, et al. (1997) Somatic inactivation of the VHL gene in Von Hippel-Lindau disease tumors. Am J Hum Genet; 60(4): p. 765-71.
112. Herman J G, Umar A, Polyak K, Graff J R, Ahuja N, et al. (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci U S A; 95(12): p. 6870-5.

113. Geisler J P, Rathe J A, Manahan K J, and Geisler H E. (2003) Methylation: a second hit in the two-hit hypothesis. Eur J Gynaecol Oncol; 24(5): p. 361.
114. Myohanen S K, Baylin S B, and Herman J G. (1998) Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia. Cancer Res; 58(4): p. 591-3.
115. Holliday R. (1987) The inheritance of epigenetic defects. Science; 238(4824): p. 163-70.
116. Chan T L, Yuen S T, Kong C K, Chan Y W, Chan A S, et al. (2006) Heritable germline epimutation of MSH2 in a family with hereditary nonpolyposis colorectal cancer. Nat Genet; 38(10): p. 1178-83.
117. Hitchins M P, Wong J J, Suthers G, Suter C M, Martin D I, et al. (2007) Inheritance of a cancer-associated MLH1 germ-line epimutation. N Engl J Med; 356(7): p. 697-705.
118. Morak M, Koehler U, Schackert H K, Steinke V, Royer-Pokora B, et al. Biallelic MLH1 SNP cDNA expression or constitutional promoter methylation can hide genomic rearrangements causing Lynch syndrome. J Med Genet; 2011: p. 28.
119. Suter C M, Martin D I, and Ward R L. (2004) Germline epimutation of MLH1 in individuals with multiple cancers. Nat Genet; 36(5): p. 497-501.
120. Gosden R G and Feinberg A P. (2007) Genetics and epigenetics--nature's pen-and-pencil set. N Engl J Med; 356(7): p. 731-3.
121. Creusot F, Acs G, and Christman J K. (1982) Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. J Biol Chem; 257(4): p. 2041-8.
122. Plagemann P G, Behrens M, and Abraham D. (1978) Metabolism and cytotoxicity of 5-azacytidine in cultured Novikoff rat hepatoma and P388 mouse leukemia cells and their enhancement by preincubation with pyrazofurin. Cancer Res; 38(8): p. 2458-66.
123. Li L H, Olin E J, Buskirk H H, and Reineke L M. (1970) Cytotoxicity and mode of action of 5-azacytidine on L1210 leukemia. Cancer Res; 30(11): p. 2760-9.
124. Santi D V, Norment A, and Garrett C E. (1984) Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. Proc Natl Acad Sci U S A; 81(22): p. 6993-7.
125. Michalowsky L A and Jones P A. (1987) Differential nuclear protein binding to 5-azacytosine-containing DNA as a potential mechanism for 5-aza-2'-deoxycytidine resistance. Mol Cell Biol; 7(9): p. 3076-83.
126. Wilson V L, Jones P A, and Momparler R L. (1983) Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2'-deoxycytidine as a possible mechanism of chemotherapeutic action. Cancer Res; 43(8): p. 3493-6.
127. Momparler R L, Bouchard J, and Samson J. (1985) Induction of differentiation and inhibition of DNA methylation in HL-60 myeloid leukemic cells by 5-AZA-2'-deoxycytidine. Leuk Res; 9(11): p. 1361-6.
128. Oka M, Meacham A M, Hamazaki T, Rodic N, Chang L J, et al. (2005) De novo DNA methyltransferases Dnmt3a and Dnmt3b primarily

mediate the cytotoxic effect of 5-aza-2'-deoxycytidine. Oncogene; 24(19): p. 3091-9.
129. Al-Romaih K, Sadikovic B, Yoshimoto M, Wang Y, Zielenska M, et al. (2008) Decitabine-induced demethylation of 5' CpG island in GADD45A leads to apoptosis in osteosarcoma cells. Neoplasia; 10(5): p. 471-80.
130. Billam M, Sobolewski M D, and Davidson N E. (2009) Effects of a novel DNA methyltransferase inhibitor zebularine on human breast cancer cells. Breast Cancer Res Treat; 120(3): p. 581-92.
131. Hsi L C, Xi X, Wu Y, and Lippman S M. (2005) The methyltransferase inhibitor 5-aza-2-deoxycytidine induces apoptosis via induction of 15-lipoxygenase-1 in colorectal cancer cells. Mol Cancer Ther; 4(11): p. 1740-6.
132. Momparler R L, Bouffard D Y, Momparler L F, Dionne J, Belanger K, et al. (1997) Pilot phase I-II study on 5-aza-2'-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs; 8(4): p. 358-68.
133. Lyons J, Bayar E, Fine G, McCullar M, Rolens R, et al. (2003) Decitabine: development of a DNA methyltransferase inhibitor for hematological malignancies. Curr Opin Investig Drugs; 4(12): p. 1442-50.
134. Reu F J, Bae S I, Cherkassky L, Leaman D W, Lindner D, et al. (2006) Overcoming resistance to interferon-induced apoptosis of renal carcinoma and melanoma cells by DNA demethylation. J Clin Oncol; 24(23): p. 3771-9.
135. Borden E C. (2007) Augmentation of effects of interferon-stimulated genes by reversal of epigenetic silencing: potential application to melanoma. Cytokine Growth Factor Rev; 18(5-6): p. 491-501.
136. Balch C, Yan P, Craft T, Young S, Skalnik D G, et al. (2005) Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. Mol Cancer Ther; 4(10): p. 1505-14.
137. Kantarjian H, Issa J P, Rosenfeld C S, Bennett J M, Albitar M, et al. (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer; 106(8): p. 1794-803.
138. Weidle U H and Grossmann A. (2000) Inhibition of histone deacetylases: a new strategy to target epigenetic modifications for anticancer treatment. Anticancer Res; 20(3A): p. 1471-85.
139. Konstantinopoulos P A, Karamouzis M V, and Papavassiliou A G. (2007) Focus on acetylation: the role of histone deacetylase inhibitors in cancer therapy and beyond. Expert Opin Investig Drugs; 16(5): p. 569-71.
140. Marks P A, Richon V M, Breslow R, and Rifkind R A. (2001) Histone deacetylase inhibitors as new cancer drugs. Curr Opin Oncol; 13(6): p. 477-83.
141. Tsuji N, Kobayashi M, Nagashima K, Wakisaka Y, and Koizumi K. (1976) A new antifungal antibiotic, trichostatin. J Antibiot (Tokyo); 29(1): p. 1-6.
142. Yoshida M, Kijima M, Akita M, and Beppu T. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem; 265(28): p. 17174-9.

143. Roh M S, Kim C W, Park B S, Kim G C, Jeong J H, et al. (2004) Mechanism of histone deacetylase inhibitor Trichostatin A induced apoptosis in human osteosarcoma cells. Apoptosis; 9(5): p. 583-9.
144. Shankar S and Srivastava R K. (2008) Histone deacetylase inhibitors: mechanisms and clinical significance in cancer: HDAC inhibitor-induced apoptosis. Adv Exp Med Biol; 615: p. 261-98.
145. Jang E R, Lim S J, Lee E S, Jeong G, Kim T Y, et al. (2004) The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor alpha-negative breast cancer cells to tamoxifen. Oncogene; 23(9): p. 1724-36.
146. Watanabe K, Okamoto K, and Yonehara S. (2005) Sensitization of osteosarcoma cells to death receptor-mediated apoptosis by HDAC inhibitors through downregulation of cellular FLIP. Cell Death Differ; 12(1): p. 10-8.
147. Yang X, Phillips D L, Ferguson A T, Nelson W G, Herman J G, et al. (2001) Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. Cancer Res; 61(19): p. 7025-9.
148. Dannenberg L O and Edenberg H J. (2006) Epigenetics of gene expression in human hepatoma cells: expression profiling the response to inhibition of DNA methylation and histone deacetylation. BMC Genomics; 7: p. 181.
149. Bjornsson H T, Fallin M D, and Feinberg A P. (2004) An integrated epigenetic and genetic approach to common human disease. Trends Genet; 20(8): p. 350-8.
150. Morgan H D, Santos F, Green K, Dean W, and Reik W. (2005) Epigenetic reprogramming in mammals. Hum Mol Genet; 14 Spec No 1(14): p. R47-58.
151. Okano M, Bell D W, Haber D A, and Li E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell; 99(3): p. 247-57.
152. Shen H, Wang L, Spitz M R, Hong W K, Mao L, et al. (2002) A novel polymorphism in human cytosine DNA-methyltransferase-3B promoter is associated with an increased risk of lung cancer. Cancer Res; 62(17): p. 4992-5.
153. Wang L, Rodriguez M, Kim E S, Xu Y, Bekele N, et al. (2004) A novel C/T polymorphism in the core promoter of human de novo cytosine DNA methyltransferase 3B6 is associated with prognosis in head and neck cancer. Int J Oncol; 25(4): p. 993-9.
154. Zhao H, Du W, Gu D, Wang D, Xue F, et al. (2009) DNMT3B 579G>T promoter polymorphism and the risk for idiopathic thrombocytopenic purpura in a Chinese population. Acta Haematol; 122(1): p. 31-5.
155. Gatto S, Ragione F D, Cimmino A, Strazzullo M, Fabbri M, et al. (2010) Epigenetic alteration of microRNAs in DNMT3B-mutated patients of ICF syndrome. Epigenetics; 5(5): p. 5.
156. Jin B, Tao Q, Peng J, Soo H M, Wu W, et al. (2008) DNA methyltransferase 3B (DNMT3B) mutations in ICF syndrome lead to altered epigenetic modifications and aberrant expression of genes regulating development, neurogenesis and immune function. Hum Mol Genet; 17(5): p. 690-709.

157. Jones J S, Amos C I, Pande M, Gu X, Chen J, et al. (2006) DNMT3b polymorphism and hereditary nonpolyposis colorectal cancer age of onset. Cancer Epidemiol Biomarkers Prev; 15(5): p. 886-91.
158. Hegde M R and Roa B B. (2009) Genetic testing for hereditary nonpolyposis colorectal cancer (HNPCC). Curr Protoc Hum Genet; Chapter 10(10): p. Unit 10 12.
159. Reeves S G, Rich D, Meldrum C J, Colyvas K, Kurzawski G, et al. (2008) IGF1 is a modifier of disease risk in hereditary non-polyposis colorectal cancer. Int J Cancer; 123(6): p. 1339-43.
160. Talseth B A, Ashton K A, Meldrum C, Suchy J, Kurzawski G, et al. (2008) Aurora-A and Cyclin D1 polymorphisms and the age of onset of colorectal cancer in hereditary nonpolyposis colorectal cancer. Int J Cancer; 122(6): p. 1273-7.
161. Roll J D, Rivenbark A G, Jones W D, and Coleman W B. (2008) DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. Mol Cancer; 7(15): p. 15.
162. Feinberg A P, Gehrke C W, Kuo K C, and Ehrlich M. (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res; 48(5): p. 1159-61.
163. Stresemann C, Bokelmann I, Mahlknecht U, and Lyko F. (2008) Azacytidine causes complex DNA methylation responses in myeloid leukemia. Mol Cancer Ther; 7(9): p. 2998-3005.
164. Wong L H and Choo K H. (2004) Evolutionary dynamics of transposable elements at the centromere. Trends Genet; 20(12): p. 611-6.
165. Yoder J A, Walsh C P, and Bestor T H. (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet; 13(8): p. 335-40.
166. Yang A S, Estecio M R, Doshi K, Kondo Y, Tajara E H, et al. (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res; 32(3): p. e38.
167. Lim H W, Iwatani M, Hattori N, Tanaka S, Yagi S, et al. (2009) Resistance to 5-aza-2'-deoxycytidine in Genic Regions Compared to Non-genic Repetitive Sequences. J Reprod Dev; 9: p. 9.
168. Gowher H and Jeltsch A. (2002) Molecular enzymology of the catalytic domains of the Dnmt3a and Dnmt3b DNA methyltransferases. J Biol Chem; 277(23): p. 20409-14.
169. Chodavarapu R K, Feng S, Bernatavichute Y V, Chen P Y, Stroud H, et al. (2010) Relationship between nucleosome positioning and DNA methylation. Nature; 466(7304): p. 388-92.
170. Weber B, Kimhi S, Howard G, Eden A, and Lyko F. (2010) Demethylation of a LINE-1 antisense promoter in the cMet locus impairs Met signalling through induction of illegitimate transcription. Oncogene: p. 1-10.
171. Laurenzana A, Petruccelli L A, Pettersson F, Figueroa M E, Melnick A, et al. (2009) Inhibition of DNA methyltransferase activates tumor necrosis factor alpha-induced monocytic differentiation in acute myeloid leukemia cells. Cancer Res; 69(1): p. 55-64.
172. Bender C M, Gonzalgo M L, Gonzales F A, Nguyen C T, Robertson K D, et al. (1999) Roles of cell division and gene transcription in the methylation of CpG islands. Mol Cell Biol; 19(10): p. 6690-8.

173. Cheng J C, Weisenberger D J, Gonzales F A, Liang G, Xu G L, et al. (2004) Continuous zebularine treatment effectively sustains demethylation in human bladder cancer cells. Mol Cell Biol; 24(3): p. 1270-8.
174. Wijermans P W, Krulder J W, Huijgens P C, and Neve P. (1997) Continuous infusion of low-dose 5-Aza-2'-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. Leukemia; 11 Suppl 1(1): p. S19-23.
175. Vishwanatha J K, Salazar E, and Gopalakrishnan V K. (2004) Absence of annexin I expression in B-cell non-Hodgkin's lymphomas and cell lines. BMC Cancer; 4(8): p. 8.
176. Kawakami K, Yamamura S, Hirata H, Ueno K, Saini S, et al. (2010) Secreted frizzled-related protein-5 (sFRP-5) is epigenetically downregulated and functions as a tumor suppressor in kidney cancer. Int J Cancer; 2010: p. 25.
177. Dannenberg L O and Edenberg H J. (2006) Epigenetics of gene expression in human hepatoma cells: expression profiling the response to inhibition of DNA methylation and histone deacetylation. BMC Genomics; 7(181): p. 181.
178. Coombes M M, Briggs K L, Bone J R, Clayman G L, El-Naggar A K, et al. (2003) Resetting the histone code at CDKN2A in HNSCC by inhibition of DNA methylation. Oncogene; 22(55): p. 8902-11.
179. Feinberg A P. (2001) Methylation meets genomics. Nat Genet; 27(1): p. 9-10.
180. Ding X, Wang Y, Zhang D, Wang Y, Guo Z, et al. (2008) Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2'-deoxycytidine and trichostatin A. Theriogenology; 70(4): p. 622-30.
181. Zhang X M, Li Q M, Su D J, Wang N, Shan Z Y, et al. (2010) RA induces the neural-like cells generated from epigenetic modified NIH/3T3 cells. Mol Biol Rep; 37(3): p. 1197-202.
182. Deng G, Kakar S, Okudiara K, Choi E, Sleisenger M H, et al. (2009) Unique methylation pattern of oncostatin m receptor gene in cancers of colorectum and other digestive organs. Clin Cancer Res; 15(5): p. 1519-26.
183. Gupta R A, Shah N, Wang K C, Kim J, Horlings H M, et al. (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature; 464(7291): p. 1071-6.
184. Matousova M, Votruba I, Otmar M, Tloustova E, Gunterova J, et al. (2011) 2 -Deoxy-5,6-dihydro-5-azacytidine - a less toxic alternative of 2 -deoxy-5-azacytidine: a comparative study of hypomethylating potential. Epigenetics; 6(6): p. 6.
185. Chen M Y, Liao W S, Lu Z, Bornmann W G, Hennessey V, et al. (2011) Decitabine and suberoylanilide hydroxamic acid (SAHA) inhibit growth of ovarian cancer cell lines and xenografts while inducing expression of imprinted tumor suppressor genes, apoptosis, G2/M arrest, and autophagy. Cancer; (16): p. 26073.
186. Flatmark K, Nome R V, Folkvord S, Bratland A, Rasmussen H, et al. (2006) Radiosensitization of colorectal carcinoma cell lines by histone deacetylase inhibition. Radiat Oncol; 1: p. 25.

187. Ramsahoye B H. (2002) Measurement of genome wide DNA methylation by reversed-phase high-performance liquid chromatography. Methods; 27(2): p. 156-61.
188. Li L C and Dahiya R. (2002) MethPrimer: designing primers for methylation PCRs. Bioinformatics; 18(11): p. 1427-31.

Chapter 8 - Appendices

8.1 – Epimutations Inheritance and Causes of Aberrant DNA Methylation in Cancer

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Epimutations, Inheritance and Causes of Aberrant DNA Methylation in Cancer

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Abstract

Epigenetic aberrations such as global hypomethylation and gene-specific hypermethylation are key events that underlie tumour development. Such scenarios are brought about by the loss of control of methylation patterns which typically are reversed in neoplasia in comparison to normal states. Despite the methylation process being termed epigenetic, suggesting that it is not a heritable condition, there is strong evidence in mouse models suggesting that epimutations within the germline may provide a mechanism through which methylation variations can be transmissible to offspring. The first half of the review will focus on the nature of methylation-induced gene silencing and transmission of this information through the germline. The latter half will focus on the cause of aberrant DNA methylation.

Introduction

In normal cells, repetitive elements such as long interspersed nucleotide elements (LINE), Alu repeats and satellite sequences, which make up almost half of the entire genome, are methylated. As this contributes largely to the level of global methylation, it is no surprise that these regions are the most drastically affected by hypomethylation, and the stability that the methylation once conferred to the chromosomes is lost. Supporting this is strong evidence to show that global hypomethylation plays a crucial role in causing genomic instability in colorectal carcinogenesis [1]. Such hypomethylation is observed in cancer cells and can be used as an indicator of genomic methylation levels [2]. Alternatively, gene specific hypermethylation is another mechanism which can initiate carcinogenesis. This mechanism of gene silencing is demonstrated by the correlation of methylated promoters with a subsequent decrease of corresponding gene expression. Some examples of genes methylated in cancer are summarised in Table 1. Co-existence of global hypomethylation and gene-specific hypermethylation is common in cancer and will be discussed in more detail later in this review.

Knudson's two-hit hypothesis [3] requires that both alleles of a tumour-suppressing gene be altered for disease progression to occur. Germline mutations commonly represent the first hit of one allele, whilst the second hit typically arises from a sporadic mutation or loss of heterozygosity that affects the second allele (Figure 1a). With the increasing detection of methylated promoters, refinements to Knudson's hypothesis can be made to accommodate epigenetic silencing. The MLH1 gene is widely studied and will be used in the following examples. One such scenario of epigenetic silencing includes methylation acting as the second hit, in unison with a pre-existing mutation on the second

Table 1. Genes frequently found methylated in bowel, endometrial, urothelial and breast tumours

Tumour location	Genes methylated	Reference
bowel	MLH1	11, 12
	p14	11, 12
	p16	11, 12
	APC	11, 12
	MGMT	12
	RASSF1A	12
	CDH1	12
endometrial	p16	13
	MLH1	
urothelial	p14	14
	p16	
	E-Cadherin (CDH1)	
	GSTP1	
breast	BRCA1	15
	COX2	16
	E-Cadherin (CDH1)	17
	HIC1	18

allele (Figure 1b) and has been detected on genes such as RB1 [4], VHL [5], MLH1 [6] and BRCA1 [7]. A typical example of this is evident in the colorectal cancer cell line HCT116, which has a truncating mutation in one allele of the p16 gene. The wild-type allele however is subjected to methylation whilst the mutated allele remains unmethylated, showing how co-operatively these two mechanisms can silence genes [8]. Thirdly is

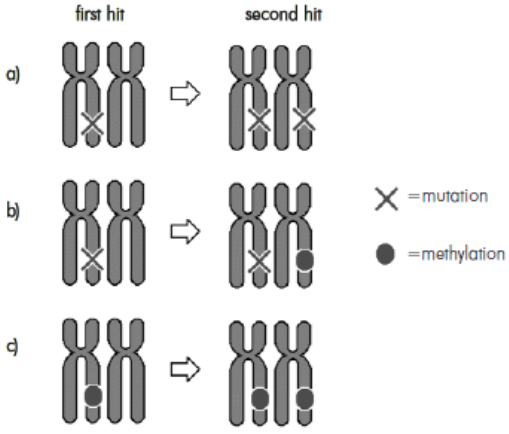

Fig. 1. Alternate mechanisms of Knudson's two-hit hypothesis. Diagram a) illustrates the original hypothesis in which two 'hits" affect both alleles of a particular gene. Diagram b) demonstrates methylation occurring as the second hit in unison with a pre-existing mutation. Diagram c) represents an individual with a soma-wide epimutation. The second allele can then also be lost due to methylation

a mechanism in which both alleles of a gene become deactivated by methylation. Sporadic cases of colon cancer are frequently the result of methylated MLH1 [9]. Methylation may even act as the first and second hits as illustrated in Figure 1c. As will be discussed shortly, certain individuals possess a silenced MLH1 allele in all cells of the body, and a second event will disrupt the wild-type allele in carcinomas [10].

Inheritance of epimutations

Epimutations have been shown to be transmitted clonally, and the thought that an epimutation can be transferred through the germline has received increasing attention in past years.

Roemer et al. [19] demonstrated that they could induce altered gene expression in mice by nuclear transfer with another cell of different genotype. In doing so, this induced methylation of two genes: major urinary protein (*Mup*) and olfactory marker protein (*Omp*). These changes were found to still be in place when the mice reached adulthood, and intriguingly the offspring were found to have increased methylation of the two genes when compared with normal controls. This was the first documented example of epigenetic inheritance, and raised questions as to whether a similar event would be possible in humans.

Ideal candidates were identified by screening populations of people who had the characteristics of a disease, yet lacked any mutation in the genes associated with the disease. Three groups which have done this recognised individuals who were mutation negative for genes associated with hereditary non-polyposis colorectal cancer (HNPCC), yet displayed phenotypic similarities to a person with the condition [10, 20-22]. These studies have identified several individuals that show mono-allelic methylation of the MLH1 allele in peripheral blood [20] and additionally in buccal mucosa and hair follicles [10, 21, 22]. As these changes were present soma-wide, they are all indicative of a parental germline change in MLH1 (inherited epimutation) or an event soon after fertilization. Subsequently, the next task was to ascertain whether the methylated allele could be transmitted to offspring and establish a mono-allelic methylation pattern. The transmission of affected alleles from parents to offspring can be monitored by detecting the presence of a polymorphism within the MLH1 promoter. In one family it was found that the methylated paternal allele (present in patient TT) had been passed to a daughter, but it did not attract the methylation as it had in the parent [10]. In a second unrelated family, a male (patient ST) carried a methylated maternal allele, but the allele was not silenced in the mother, or siblings who inherited the

corresponding allele [22]. Details of a third family are less detailed; two children of an affected mother (patient VT) possessed normal MLH1 alleles but the status of a third child and the father were not known [10]. Despite neither patient VT nor TT carrying a mutation within a known HNPCC associated gene, they were part of families with histories of the disease. This may suggest that more complex genetic interactions or a gene not linked to MLH1 was causative of their condition rather than a distinct mutation.

The most compelling evidence for transmission of epimutated alleles is shown by the analysis of patient TT's spermatozoa. Following PCR, cloning and hybridization with a methylation-specific probe, 5 of 526 colonies showed a methylated MLH1 promoter. Affected spermatozoa could potentially be transmitted to offspring, rendering them more susceptible to developing disease. Despite only 1% of spermatozoa being affected, this revealed that a) the majority of methylation present is removed during meiosis, and b) that transmission to offspring would be rare, but possible. Cases reported so far have yet to identify the transmission of a methylated allele, so the evidence would support an early epimutational event rather than an inherited predisposition as the cause, although the number of cases studied is small and parental genetic information cannot always be obtained. If indeed the methylated allele was transmitted in that state, the methylation may even be removed early in embryogenesis in the wave of demethylation [23]. Future work may prove epigenetic inheritance using other genes that are commonly affected in disease.

Aberrant methylation patterns

A common characteristic of cancer cells is a reversal of normal methylation patterns; a high level of methylation is observed in specific promoter regions and a global decrease in genomic methylation [24]. It would appear that these features are a cause rather than a consequence of the cancer as alterations can be identified in the early stages of cancer development. For this reason, the mechanism which regulates the methylation process has been highly sought after, yet remains elusive. Several facets which may affect the normal functioning of methylation controlled gene regulation will be discussed here.

DNMT over-expression

Among the most common explanations for the disturbance of regular methylation patterns is the up-regulation of the methyltransferase enzymes. On numerous occasions it has been demonstrated that DNMT levels are elevated in several diverse forms of cancer such as leukaemia [25], endometrial cancer [26] and lung cancer [27]. It could be argued that the down-regulation of maintenance methylation by DNMT1 may lead to hypomethylation, whilst simultaneous up-regulation of the de-novo methyltransferases DNMT3a and 3b could account for the increase in aberrantly methylated promoters. Kimura et al. [28] showed that DNMT1 maintenance methyltransferase expression was not correlated with the extent of DNA hypomethylation in transitional cell carcinomas; however, there was a decrease of DNMT1 expression relative to cell proliferation. Addressing the hypermethylation of certain promoters, it was shown that DNMT3b levels were higher than corresponding normal tissue, although DNMT3a levels were not. While it seems a likely explanation in this scenario, it is not always so straightforward. Observations include simultaneous up- and down-regulation of de novo (DNMT3a and 3b) and maintenance (DNMT1) transferases, and the level of expression of the methyltransferases is sometimes variable within the same cell type [29].

Doubt also surrounds whether over-expression of methyltransferases is responsible for hypermethylation. Eads et al. [30] showed that the expression levels of DNMT1, 3a and 3b did not correlate with the frequency or extent of hypermethylation of APC, ESR1, p16 or MLH1 in colorectal adenocarcinomas. Whilst the methyltransferases were up-regulated when normalised with β-actin and an RNA polymerase large subunit, they were not significantly up-regulated when normalised with proliferation-dependant H4F2 or PCNA. This suggests that although the methyltransferase levels appear to be increased in many cell types, they may in fact not be when they are normalised with other proliferation-dependant genes.

Amid the evidence of altered levels and variations of expression levels, it would not seem logical that simple up-regulation or down-regulation of one form of methyltransferase would cause site-specific hypermethylation in parallel with a global decrease in methylation, but rather a co-ordinated alteration between de novo and maintenance forms to explain the aberrant methylation state.

Subtle CpG island differences

It is evident that some CpG islands are more often affected than others by methylation, supported by the observation that a cluster of genes is frequently hypermethylated in several types of cancer cells [24]. It is no surprise that these genes are heavily involved

in the maintenance of genomic integrity, tumour suppression and metastasis, and that these promoters are unaffected in normal tissue. An example of such is the well characterised MLH1 gene in bowel cancer syndromes. This gene is one of four genes which confer a higher susceptibility to HNPCC, yet MLH1 is the only one of the four which becomes methylated.

It would seem reasonable to suggest that some CpG islands may be more likely to succumb to methylation based on CpG island size, GC content, CpG frequency, chromosomal location or promoter association. An experiment by Feltus et al. [31] in 2003 examined the methylation state of several CpG islands in cells over-expressing DNMT1. The majority of the CpG islands tested were resistant to methylation, but a small proportion (3.8%) were found to be hypermethylated by DNMT1. Using this information they identified seven sequence patterns that were capable of discriminating between methylation prone and resistant islands with a success rate of 87%. These sequences would appear to confer some kind of susceptibility or resistance to methylation, possibly similar to the situation in which a non-methylated imprinted allele is resistant to methylation.

In this study, the number of methylated CpG islands may have been biased, due to the over-expression of the maintenance methyltransferase rather than the de novo forms. Therefore the hypermethylated regions in the study are in essence DNMT1 susceptible regions, and it is possible that the number of methylated islands would be higher if de novo methyltransferases were over-expressed. Nonetheless, this study has shown that no particular characteristic such as CpG frequency or island size affects methylation susceptibility, but more so a subset of DNA sequences which may attract or repel methylation. In terms of initiating hypermethylation, these sequences alone will not define the methylation status of a particular gene, as non-methylated regions in normal tissue will also have the same sequence.

DNA demethylation

The issue of DNA demethylation is a major unknown in the field of epigenetics. There has been some debate that still continues over the presence and existence of DNA demethylating enzymes. A demethylating enzyme has been uncovered, but this acts on histones rather than DNA [32]. The most controversy surrounds the MBD2 gene, with one group claiming it has DNA demethylating properties [33-35] whilst others continue to find that it acts as a transcriptional repressor [36-38]. Much work has been performed involving the gene and there are numerous reports that support each side of the debate that will not be discussed here. For details of these, see references [33-38], in particular reference 33, in which supporters of MBD2's demethylase action address issues raised by others suggesting MBD2 is a transcriptional repressor.

At present, the identification of a bona fide demethylase is yet to occur; however, should a candidate be recognised, it will no doubt trigger enormous interest in the particular gene's role in cancer.

Dietary factors, including folate metabolism

There are vast amounts of evidence that nutrition obtained from dietary components has a major influence on individual health status, and there are at least two pathways with which nutrient intake can affect methylation, as reviewed in [39], and more recently [40]. The first of these simply states that the supply of nutrients affects the supply of methyl groups required for methylation. Numerous dietary components are known to influence DNA methylation status, and folate, choline and vitamin B_{12} feature highly in the literature. Various forms of folate are converted into intermediates that are ultimately converted to S-adenosyl-methionine, the chemical substrate with which the methyltransferase enzymes obtain methyl groups for attachment to the DNA [41]. The precise role of folate and the effects of its absence are complex (for a review see reference 42), and its importance is due to its function as a precursor methyl-donor. Vitamin B_{12} is a co-factor for many enzymatic processes leading to the methylation of DNA. Rats fed a diet deficient in B_{12}, but not severe enough to cause illness, were observed to have hypomethylated genomic DNA in colon tissue in comparison to appropriate controls, illustrating that a vitamin deficient diet can restrict DNA methylation.

The second mechanism in which diet can influence methylation relies on the effects of trace dietary components interfering with methyltransferase processes. A selenium deficiency has been shown to cause DNA hypomethylation in rat colon DNA, while prolonged cadmium exposure also initiates hypomethylation followed by hypermethylation, suggesting that a feedback mechanism is involved [43]. In the same study, it was suggested that cadmium inhibited the methyltransferases via an interaction with the DNA binding domain rather than the catalytic domain. Nickel [44] and alcohol [45] have also been observed to affect DNMT activity, although the precise mechanism of this is not understood.

Methylation spreading

The expansion of existing methylation to cover neighbouring non-methylated sites is another hypothesis to explain aberrantly silenced genes.

An experiment by Tollefsbol & Hutchinson [46] has shown that using synthetic oligonucleotides, pre-existing methylation was able to spread to neighbouring CpG islands. It was found that this pseudo-DNA with partial CpG methylation was more likely to undergo de novo methylation on non-affected CpGs than a control without pre-existing methylation. This would suggest that the methylation was required for the methyltransferases to recognise the DNA and spread the methylation. Furthermore, mammalian methyltransferases were the only proteins necessary to induce this state, eliminating the notion that other factors are required for the expansion.

These results provide evidence which supports the spreading of methylation from ordinarily methylated DNA regions to areas which would not usually be methylated. The precise role that this method plays in disease initiation is not known, and methylation spreading has a weaker justification for the aberrant methylation observed in cancer, particularly global hypomethylation. However, if the spreading of methylation to normally unaffected regions occurs in conjunction with another mechanism such as down-regulation of maintenance methylation, it could provide a clearer pathway in which aberrant disease-causing methylation patterns arise.

Conclusion

Epimutation of several genes has been shown to cause disease, and is equivalent to mutations within the same gene, but is a reversible trait. Inheritance of epimutations is an interesting facet of genetics, which may possibly play a role in a small percentage of cancer cases. There is evidence of its occurrence in mammals, yet definite proof of its existence in humans is yet to be demonstrated.

With regards to the regulation of methylation, the five mechanisms discussed each provide a possible explanation for aberrant DNA methylation. However, there does not seem to be one theory that can conclusively account for the abnormal methylation patterns in cancer, namely localised hypermethylation and genome-wide hypomethylation. A combination of events such as any of those discussed above with other environmental and other unknown genetic factors may have a cumulative effect on abnormal DNA methylation.

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References

1. Matsuzaki K, Deng G, Tanaka H, Kakar S, Miura S and Kim YS The relationship between global methylation level, loss of heterozygosity, and microsatellite instability in sporadic colorectal cancer. Clin Cancer Res 2005; 11 (24 Pt 1): 8564-8569.
2. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M and Laird PW. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acid Res 2005; 33: 6823-6836.
3. Knudson A. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA 1971; 68: 820-823.
4. Ohtani-Fujita N, Dryja TP, Rapaport JM, Fujita T, Matsumura S, Ozasa K, Watanabe Y, Hayashi K, Maeda K, Kinoshita S, Matsumura T, Ohnishi Y, Hotta Y, Takahashi R, Kato MV, Ishizaki K, Sasaki MS, Horsthemke B, Minoda K and Sakai T. Hypermethylation in the retinoblastoma gene is associated with unilateral, sporadic retinoblastoma. Cancer Genet Cytogenet 1997; 98: 43-49.
5. Prowse AH, Webster AR, Richards FM, Richard S, Olschwang S, Resche F, Affara NA and Maher ER. Somatic inactivation of the VHL gene in Von Hippel-Lindau disease tumours. Am J Hum Genet 1997; 60: 765-771.
6. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA and Baylin SB. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 1998; 95: 6870-6875.
7. Geisler JP, Rathe JA, Manahan KJ and Geisler HE. Methylation: a second hit in the two-hit hypothesis. Eur J Gynaecol Oncol 2003; 24: 361.
8. Myohanen SK, Baylin SB and Herman JG. Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia. Cancer Res 1998; 58: 591-593.
9. Furukawa T, Konishi F, Masubuchi S, Shitoh K, Nagai H and Tsukamoto T. Densely methylated MLH1 promoter correlates with decreased mRNA expression in sporadic colorectal cancers. Genes Chromosomes Cancer 2002; 35: 1-10.
10. Suter CM, Martin DI and Ward RL. Germline epimutation of MLH1 in individuals with multiple cancers. Nat Genet 2004; 36: 497-501.
11. Brucher BL, Geddert H, Langner C, Hofler H, Fink U, R Siewert J and Sarbia M. Hypermethylation of hMLH1, HPP1, p14 (ARF), p16 (INK4A) and APC in primary adenocarcinomas of the small bowel. Int J Cancer 2006; [Epub ahead of print].
12. Lee S, Hwang KS, Lee HJ, Kim, JS and Kang GH. Aberrant CpG island hypermethylation of multiple genes in colorectal neoplasia. Lab Invest 2004; 84: 884-893.
13. Yanokura M, Banno K, Susumu N, Kawaguchi M, Kuwabara Y, Tsukazaki K and Aoki D. Hypemethylation in the p16 promoter region in the carcinogenesis of endometrial cancer in Japanese patients. Anticancer Res 2006; 26: 851-856.
14. Dhawan D, Hamdy F, Rehman I, Patterson J, Cross S, Feeley K, Stephenson Y, Meuth M and Catto J. Evidence for the early onset of aberrant promoter methylation in urothelial carcinoma. J Pathol 2006; [Epub ahead of print].
15. Bianco T, Chenevix-Trench G, Walsh DC, Cooper JE and Dobrovic A. Tumour specific distribution of BRCA1 promoter region methylation supports a pathogenic role in breast and ovarian cancer. Carcinogenesis 2000; 21: 147-151.
16. Ma X, Yang Q, Wilson KT, Kundu N, Meltzer SJ and Fulton AM. Promoter methylation regulates cyclooxygenase expression in breast cancer. Breast Cancer research 2004; 6: R316-R321.
17. Cheng CW, Wu PE, Yu JC, Huang CS, Yue CT, Wu CW and Shen CY. Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two hit hypothesis of tumour suppressor gene. Oncogene 2001; 20: 3814-3823.
18. Fujii H, Biel MA, Zhou W, Weitzman SA, Baylin SB and Gabrielson F. Methylation of the HIC-1 candidate tumour

suppressor gene in human breast cancer. Oncogene 1998; 16: 2159-2164.
19. Roemer I, Reik W, Dean W and Klose J. Epigenetic Inheritance in the mouse. Curr Biol 1997; 7: 277-280.
20. Gazzoli I, Loda M, Garber J, Syngal and Kolodner RD. A hereditary non polyposis colorectal carcinoma case associated with hyper methylation of the MLH1 gene in normal tissue and loss of heterozygosity of the unmethylated allele in the resulting microsatellite instability-high tumour. Cancer Res 2002; 62: 3925-3928.
21. Miyakura Y, Sugano K, Akasu T, Yoshida T, Maekawa M, Saitoh S, Sasaki H, Nomizu T, Konishi F, Fujita S, Moriya Y and Nagai H. Extensive but hemiallelic methylation of hMLH1 promoter region in early-onset sporadic colon cancers with microsatellite instability. Clin Gastroenterol Hepatol 2004; 2: 147-156.
22. Hitchins M, Williams R, Cheong K, Halani N, Ap Lin V, Packham D, Ku S, Buckle A, Hawkins N, Burn J, Gallinger S, Goldblatt J, Kirk J, Tomlinson I, Scott R, Spigelman A, Suter C, Martin D, Suthers G and Ward R. MLH1 germline epimutations as a factor in Hereditary non polyposis colorectal cancer. Gastroenterology 2005; 129: 1392-1399.
23. Reik W, Dean W and Walter J. Epigenetic reprogramming in mammalian development. Science 2001; 293: 1089-1093.
24. Esteller M, Corn PG, Baylin SB and Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001; 61: 3225-3229.
25. Melki JR, Warnecke P, Vincent PC and Clark SJ. Increased DNA methyltransferase expression in leukemia. Leukemia 1998; 12: 311-316.
26. Jin F, Dowdy SC, Xiong Y, Eberhardt NL, Podratz KC and Jiang SW. Up-regulation of DNA methyltransferase 3B expression in endometrial cancers. Gynecol Oncol 2005; 96: 531-538.
27. Belinsky SA, Nikula KJ, Baylin SB and Issa JP. Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. Proc Natl Acad Sci USA 1996; 93: 4045-4050.
28. Kimura F, Seifert HH, Florl AR, Santourlidis S, Steinhoff C, Swiatkowski S, Mahotka C, Gerharz CD and Schulz WA. Decrease of DNA methyltransferase 1 expression relative to cell proliferation in transitional cell carcinoma. Int J Cancer 2003; 104: 568-578.
29. De Marzo AM, Marchi VL, Yang ES, Veeraswamy R, Lin X and Nelson WG. Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. Cancer Res 1999; 59: 3855-3860.
30. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV and Laird PW. CpG island hypermethylation in human colorectal tumours is not associated with DNA methyltransferase overexpression. Cancer Res 1999; 59: 2302-2306.
31. Feltus FA, Lee EK, Costello JF, Plass C and Vertino PM. Predicting aberrant CpG island methylation. Proc Natl Acad of Sci USA 2003; 100: 12253-12258.
32. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA and Shi Y. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 2004; 119: 941-953.
33. Detich N, Theberge J and Szyf M. Promoter-specific activation and demethylation by MBD2/demethylase. J Biol Chem 2002; 277: 35791-35794.
34. Szyf M and Bhattacharya SK. Measuring DNA demethylase activity in vitro. Methods Mol Biol 2002; 200: 155-161.
35. Bhattacharya SK, Ramchandani S, Cervoni N and Szyf M. A mammalian protein with specific demethylase activity for mCpG DNA. Nature 1999; 397: 579-583.
36. Ng HH, Zhang Y, Hendrich B, Johnson, CA, Turner BM, Erdjument-Bromage H, Tempst P, Reinberg D and Bird A. The methyl-CpG binding protein MBD2 is a transcriptional repressor belonging to the MeCP1 fraction of histone deactylase complexes. Nat Genet 1999; 23: 58-61.
37. Boeke J, Ammerpohl O, Kegel S, Moehren U and Renkawitz, R. The minimal repression domain of MBD2b overlaps with the methyl-CpG-binding domain and binds directly to Sin3A. J Biol Chem 2000; 275: 34963-34967.
38. Wade PA, Gegonne A, Jones PL, Ballestar E, Aubry F and Wolffe AP. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet 1999; 23: 62-66.
39. Liu L, Wylie RC, Andrews LG and Tollefsbol TO. Aging, cancer and nutrition: the DNA methylation connection. Mech Ageing Dev 2003; 124: 989-998.
40. McCabe DC and Caudill MA. DNA methylation, genomic silencing and links to nutrition and cancer. Nutr Rev 2005; 63: 183-195.
41. Wu JC and Santi DV. On The mechanism and inhibition of DNA cytosine methyltransferases. Prog Clin Biol Res 1985; 198: 119-129.
42. Kim YI. Folate and DNA methylation: a mechanistic link between folate deficiency and colorectal cancer? Cancer Epidemiol Biomarkers Prev 2004; 13: 511-519.
43. Takiguchi M, Achanzar WE, Qu W, Li G and Waalkes MP. Effects of cadmium on DNA-(cytosine-5) methyltransferase activity and DNA methylation status during cadmium-induced cellular transformation. Exp Cell Res 2003; 286: 355-365.
44. Costa M, Davidson TL, Chen H, Ke Q, Zhang P, Yan Y, Huang and Kluz T. Nickel carcinogenesis: epigenetics and hypoxia signalling. Mutat Res 2005; 592: 79-88.
45. Bönsch D, Lenz B, Fiszer R, Frieling H, Kornhuber J and Bleich S. Lowered DNA methylatransferase (DNMT-3b) mRNA expression is associated with genomic DNA hypermethylation in patients with chronic alcoholism. In desktop folder.
46. Tollefsbol TO and Hutchison CA 3rd. Control of methylation spreading in synthetic DNA sequences by the murine DNA methyltransferase. J Mol Biol 1997; 269: 494-504.

8.2 – Detailed Methods

8.2.1 - Cell Culture

Colorectal cancer cells were grown in 1X DMEM supplemented with 10% Foetal Bovine Serum at 37°C and 5% CO_2. All cells were obtained from the Molecular Genetics Laboratory, Hunter New England Health Service, John Hunter Hospital, Newcastle, Australia, which were originally obtained from the American Type Culture Collection.

8.2.1.2 - Cell treatment with demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC)

DNA demethylation of cultured cells was induced by treatment with 5-aza-dC (Sigma-Aldrich). 10mg of 5-aza-dC was dissolved in 4382µL of 1X PBS to create a stock of 10,000µM and aliquots were frozen at -80°C until ready for use. To determine the concentration of 5-aza-dC which would induce maximum demethylation of DNA, cells were treated with increasing concentrations of the drug for 72 hours, and media was replaced every 24 hours with fresh drug containing media due to the instability of 5-aza-dC in cell culture media. The optimal concentration was determined after extraction of DNA and HPLC analysis.

8.2.1.3 - Cell treatment with Histone De-acetylase (HDAC) Inhibitor, Trichostatin A

Trichostatin A (10mg) (Sigma-Aldrich) was dissolved in 1mL 100% ethanol at a concentration of 3307nM and stored at -20ºC. Treatment of cells was performed over 72 h, to coincide with 5-aza-dC treatment, and media was replaced every 24 h due to the instability of the drugs in culture media. A final concentration of 150nM was used on HCT116 cells as this has been previously shown to induce substantial histone acetylation [186].

8.2.1.4 - Re-methylation experiments

Initially DNA and RNA were extracted from untreated cells to compare against the drug treated series of samples. Cultured cells were then grown for 72 h in the presence of 5-aza-dC (or 5-aza-dC and TSA for HCT116 cells) at appropriate concentrations. After 72 h of treatment, DNA and RNA were extracted from a fraction of the cells. The remaining fraction of cells were washed twice with 1X PBS to remove any trace of the drugs, and allowed to recover under normal incubation conditions. DNA and RNA were extracted from a fraction of the cells at every two days period after initial removal of the drug, whilst the unused fraction of cells were incubated under normal conditions until the 10th day of drug free growth. Cells were passaged as required and media was replaced at least every 48 h.

8.2.1.5 - DNA extraction from cultured cells

Growth media was removed and cells were lifted from the culture flask by incubation with 0.5% trypsin at 37°C. Trypsinised cells were then centrifuged with one volume of DMEM- media at 620 x *g* for 5 min. The supernatant was then discarded and the cell pellet was resuspended in 2mL of Nuclear Lysis Buffer, 100µL Proteinase K (20ug/ml) and 100µL 20% SDS. Cells were aspirated with an 18 gauge syringe several times to facilitate cell membrane disruption and then incubated overnight at 55°C to allow protein degradation. Following incubation, the sample was split into two 2ml screw cap tubes and 333µl of 6M NaCl solution was added to each to precipitate cellular protein. Samples were vortexed for 30s and then centrifuged at 17,000 x *g* for 30 min to pellet the insoluble protein fraction. The supernatants of corresponding samples were then combined and 2.5 volumes of ice cold ethanol was added and inverted several times. Precipitated DNA was removed with a transfer pipette into a new 1.5mL screw cap tube. DNA was washed with the addition of 70% ethanol, inverted several times and centrifuged at 17,000 x *g* for two min. The 70% ethanol wash was repeated once. Residual 70% ethanol was removed and the DNA was resuspended in an appropriate volume of 1X TE solution and incubated at 55°C to allow for dissolution, aided with occasional pipetting.

8.2.1.6 - RNA extraction

Cells for RNA extraction were removed from culture flasks in the same manner as the DNA extraction method with 0.5% trypsin and centrifugation performed in DMEM- media. The supernatant was discarded and the cell pellet was resuspended in 1mL Trizol per 10^6 cells. Cells were aspirated with an 18 gauge needle several times and incubated at room temperature for at least 5 min, or stored at 4ºC. Following incubation, 0.2mL chloroform was added and shaken vigorously for 15 seconds and incubated for three min at room temperature. Samples were then centrifuged at 12,000 x g for 15 min at 4ºC to create layers of solution. The clear upper aqueous phase was then removed to a new tube. RNA was precipitated with the addition of 0.5mL isopropanol and incubated at room temperature for 10 min. The sample was then centrifuged at 12,000 x g for 5 min to pellet the RNA. The supernatant was then discarded and the RNA pellet was washed with 75% ethanol, centrifuged at 7,500 x *g*, then dried and resuspended in 20-40µL sterile Milli-Q water and stored at -80ºC.

8.2.2 - High Performance Liquid Chromatography (HPLC)

8.2.2.1 - DNA purification

Prior to HPLC analysis, DNA was purified to eliminate any RNA which would interfere with the analysis. Approximately 50µg of DNA was treated with an RNAse Cocktail containing 2.5U/mL RNAse A and 100U/mL RNAse T1 to degrade any contaminating RNA and incubated at 37°C for 2 h. Following incubation, 1.5 volumes of phenol-chloroform was added, inverted several time to mix and centrifuged at 17,000 x *g* for 10 min to form layers of solution. The top aqueous layer containing the DNA was aspirated, and placed in a new tube. The DNA was precipitated with 100% ice cold ethanol (and 5µL Linear Acrylamide (Ambion) if a low yield is expected). The DNA was then centrifuged at 17,000 x *g* for 2 min, and then washed twice with 70% ethanol and resuspended in a small volume of Water For Injection (Astra Zeneca). DNA yield and concentration was quantified by measuring the optical density with a Varian Spectrophotometer.

8.2.2.2 - DNA Digestion

Degradation of DNA into component nucleosides was performed with a series of enzymatic digestions. 3µg of purified DNA in a 0.2mL PCR tube was heated to 100°C for 5 min then quickly placed on ice to denature the DNA to single strands. Once cooled, the DNA was digested with 1.5U of Nuclease P1 (US Biologicals) and incubated at 37°C for 16 h in a GeneAmp Thermal Cycler (Applied Biosystems). Following Nuclease P1 digestion, phosphate groups of the DNA backbone were removed with the addition of 2µl Calf Intestinal Alkaline Phosphatase (Promega) at room temperature and then incubated at 37°C for at least 2 h.

8.2.2.3 - Nucleoside Standards Preparation

Nucleoside standards were prepared to ensure correct peak identification in HPLC analysis. A Polymerase Chain Reaction was performed using conditions shown in Table 8.2.1 and 8.2.2 to generate a 703bp fragment of a CpG island within the MLH1 promoter region containing 55 CpG dinucleotides. These 55 cytosines are able to be methylated *in vitro*, and represent 24% of the total cytosine content of the PCR product.

Table 8.2.1 - *MLH1* promoter PCR mastermix components for a single reaction

Component	Volume (µL)
Water	32.5
10X PCR buffer	5
dNTPs (2.5µM)	4
$MgCl_2$ (50mM)	1.5
Forward primer (20µM)	1
Reverse primer (20µM)	1
Taq polymerase (5U/µl)	0.4
Template DNA (50ng/ul)	4

Table 8.2.2 - Uni 63 PCR Thermal cycler conditions. The annealing temperature of 63ºC was decreased by 0.5ºC each subsequent cycle in the initial 14 cycles

Temperature	Time	Cycles
94°C	5 minutes	1
94°C	30 seconds	
63°C *	45 seconds	x14
72°C	60 seconds	

94°C	30 seconds	
56°C	45 seconds	x20
72°C	60 seconds	
72°C	10 minutes	x1

8.2.2.4 - Ampure PCR product purification

This method of PCR product purification uses small magnetic beads to attract the negatively charged DNA whilst unused reagents are removed. A volume of 20µL PCR product is mixed with 36µL of Ampure beads, 100µL of 70% ethanol, mixed several times and incubated for 5 mins at room temperature. Tubes or plates were then transferred to a magnetic plate holder which attracts the magnetic beads and PCR products to the edge of the well. Liquid was then aspirated with a fine pipette and discarded. The beads were then washed twice by addition of 100µL of 70% ethanol. After the final wash, the beads were allowed to dry to remove remaining traces of ethanol. 40µL of Milli-Q was then added the elute PCR products, and 20µL was then removed to a new plate or tube.

8.2.2.5 - Methylation of cytosine with SssI Methylase

Purified products were methylated in vitro with *Sss*I Methylase (New England Biolabs) under the conditions shown below in table 8.2.3. This enzyme will attach a methyl group to cytosine residues within the 5'…CG…3' dinucleotide sequence. Samples were then incubated at 37°C for 16 h and then were purified again with the Ampure method and subjected to DNA digestion as described above.

Table 8.2.3 – *Sss*I methylase mastermix components for a single reaction

Component	Volume (µL)
DNA template	20
Reaction buffer	2.5
SAM (32nM)	1
*Sss*I (4000U/ml)	0.2

8.2.2.6 - Determination of 5-methylcytosine content with HPLC

HPLC analysis of nucleosides was performed on a Varian Star Chromatography Workstation. 3µg of digested DNA samples were injected to a Supelcosil LC-18-

DB column, where separation took place at 37°C and absorbance was monitored at 278nm (λ_{max} of 5-methylcytosine nucleoside) [187]. Elution took place over 35 min with 0.03M $NH_4H_2PO_4$ buffer with 5% methanol. Peak areas were quantified with Star Reviewer Software (Varian) and the 5-methylcytosine content was expressed as a percentage of the total cytosine pool after correction for extinction co-efficients.

$$\text{Percentage methylation} = \frac{5\text{mdC} \times 100}{(5\text{mdC} + \text{dC})}$$

8.2.3 - Expression Arrays

Based upon data generated from the analysis of global methylation levels, gene expression arrays were performed at four time points for each of the cell lines analysed. These were the untreated samples, directly after drug treatment (or Day 0 (d0) of re-methylation), four days after drug treatment (d4 of remethylation) and after 10 days of drug free growth (d10 of remethylation). RNA from these time points was used on the Illumina Human Ref 8 Beadchips using the method described below.

8.2.3.1 - RNA purification

RNA to be used for expression array analysis was desalted using the Qiagen RNeasy MinElute clean up kit. Typically, 10µl of extracted RNA was made up to 100µl with RNAse free water. 350µL of Buffer RLT was then added to the RNA, followed by 250µL of 96-100% ethanol and mixed thoroughly by pipetting. Each sample was then immediately applied to a RNeasy MinElute spin column, which was held inside a 2ml tube and centrifuged at $\geq$8000 x *g* for 15 seconds. The flow through of the spin column was discarded with the 2mL tube, and the spin column was transferred to a new 2mL tube. 500µl Buffer RPE was applied to the column, and centrifuged again at $\geq$8000 x *g* for 15 seconds. The RNA and column membrane was washed with the addition of 500µL 80% ethanol to the spin column Centrifugation was once again at $\geq$8000 x *g* for 2 min. The 2mL tube containing the flow through was discarded and the spin column was transferred to a new 2mL tube and centrifuged at full speed for 5 minutes to

remove any residual ethanol. Following this, the spin column was transferred to a new 1.5mL tube, and 20µL of RNAse free water was added. Elution was performed by centrifuging at full speed for 1 min. The purified samples were then quantitated and stored at -70ºC.

8.2.3.2 - RNA quantification

Prior to amplification, the RNA was quantified using Ribogreen reagent (Invitrogen). Samples were diluted 1:10, and 1ul of sample was added to 99µL 1X TE and 100µL of 1X Ribogreen reagent. A standard curve of increasing RNA standards was constructed using the known standards supplied with the kit, and the reagents listed in table 8.2.4. The quantification took place in 96 well light resistant plates, and measurements were taken with a FlouStar Optima Flourimeter. Unknown concentrations of RNA samples were determined automatically with software based on the equation obtained from the standard curve samples.

Table 8.2.4 - Preparation of known RNA concentrations for use in the Standard Curve

Volume TE (µl)	Volume of 2µg/mL RNA std (µL)	Volume Ribogreen (µL)	Total RNA (ng)
0	100	100	200
50	50	100	100
90	10	100	20
98	2	100	4
100	0	100	0

8.2.3.3 - Illumina TotalPrep RNA Amplification

RNA to be used on the expression arrays were treated with an Illumina TotalPrep RNA Amplification Kit (Ambion) as described in the following steps.

8.2.3.4 - Reverse Transcription to Synthesise First Strand cDNA

To synthesise first strand cDNA a mastermix was prepared at room temperature and then placed on ice using the components listed in table 8.2.5. 50-500ng of purified RNA samples in a volume of 11uL was added to 9uL of mastermix, mixed 2-3 times by pipetting and incubated at 42ºC for 2 hours. After incubation

tubes were removed and placed on ice before immediately proceeding to Second Strand cDNA synthesis.

Table 8.2.5 - Components required for a single First Strand cDNA synthesis reaction

Component	Volume (µL)
T7 Oligo d(T) primer	1
10X First Strand Buffer	2
dNTP mix	4
RNase Inhibitor	1
Array Script	1

8.2.3.5 - Second strand cDNA synthesis

A mastermix of reagents required for second strand synthesis was prepared on ice in the order listed below in table 8.2.6. The mastermix was gently vortexed before 80µL was added to each of the samples, and was mixed four times by pipetting before being placed at 16ºC in a thermal cycler PCR machine without a heated lid for 2 h. After the incubation, tubes were placed on ice before cDNA purification, which was performed immediately.

Table 8.2.6 - Components required for a single Second Strand cDNA synthesis reaction.

Component	Volume
Nuclease-Free water	63
10X Second Strand Buffer	10
dNTP mix	4
DNA Polymerase	2
RNAse H	1

8.2.3.6 - cDNA purification

Prior to amplification, the cDNA was purified from the reaction mixture. The entire reaction mixture was transferred to a 0.5mL tube, to which 250µL cDNA Binding Buffer was added and mixed 3-4 times by pipetting. The sample was then applied to a cDNA Filter Cartridge held inside a 2mL wash tube and centrifuged at 10,000 x *g* for 1 min. The flow through was discarded and 500µL of wash buffer was applied to each cDNA filter cartridge. Tubes were centrifuged at 10,000 x *g* for 1 min, the flow through was discarded and the tubes

were centrifuged again to remove any traces of the wash buffer. The cDNA filter cartridge was then transferred to a cDNA elution tube. cDNA was eluted with the addition of 10µL of nuclease-free water preheated at 55ºC. This was incubated at room temperature for 2 min before centrifugation at 10,000 x *g* for 90 s. A further 9µL of preheated water was added to the cDNA filter cartridge and centrifuged under the same conditions. Purified cDNA was then used to synthesise cRNA.

8.2.3.7 - In Vitro Transcription to Synthesise cRNA

An IVT mastermix was prepared at room temperature according to table 8.2.7. A volume of 7.5µL of mastermix was added to each cDNA sample, mixed 3-4 times by pipetting and incubated for 14 h at 37ºC in a hybridisation oven. The reaction was stopped with the addition of 75µL Nuclease free water and mixed thoroughly.

Table 8.2.7: Components required for a single cRNA synthesis reaction

Component	Volume (µL)
T7 10X Reaction Buffer	2.5
T7 Enzyme Mix	2.5
Biotin-dNTP Mix	2.5

8.2.3.8 - cRNA Purification

The newly produced cRNA was purified to remove any traces of enzymes, salts and unincorporated nucleotides from the reaction mixture. To each sample, 350µL of cRNA binding buffer was added, followed immediately by the addition of 250µL ACS reagent grade 100% ethanol and mixed three times with gentle pipetting. This mixture was then immediately applied to a cRNA Filter Cartridge and centrifuged for 1 min at 10,000 x *g*. The flow through was discarded and 650µL of Wash Buffer was added to the filter cartridge. The cRNA as washed with centrifugation at 10,000 x *g* for 1 min. The flow through was discarded and the centrifugation was repeated to remove trace amounts of wash buffer. The cRNA Filter Cartridge was then transferred to a new cRNA collection tube, and 100µL of 55ºC preheated nuclease-free water was applied to the filter. After 2 minutes of incubation at room temperature, samples were eluted by centrifugation at 10,000 x *g* for 90 s.

8.2.3.9 - Hybridisation to Illumina 8x1 Beadchips

According to the manufacturer's instructions, 750ng of cRNA in 5µL was required for hybridisation of each sample to the array. Each sample was quantified using the Ribogreen assay described earlier, with the exception that the samples were not diluted 1:10 before addition to the reaction mixture. Samples that were deemed too dilute were concentrated in a DNA SpeedVac on medium heat (40ºC-50ºC) until a volume of 5µL remained.

To each sample, 10µL of GEX-HYB was added, and preheated at 65ºC for 5 min. During this incubation, Hybridisation Chambers were assembled with the addition of 200µL GEX-HCB into each of the humidifying reservoirs. The chambers were then sealed at left at room temperature until needed. The beadchips were removed from their packaging and placed in a Hyb Chamber insert. After the 5 min incubation, tubes were allowed to cool to room temperature. 15µL of each assay sample was then dispensed to a sample port on the array, and then the Hyb Chamber inserts were inserted to the Hyb Chamber. This Hyb Chamber lid was sealed and placed on rocking platform set at speed 5, for 16-20 h overnight at 58ºC.

8.2.3.10 - Illumina 8x1 Beadchip washing, blocking and staining

Following incubation, Hyb chambers were disassembled and the cover-seal that was used to keep samples separate was removed. Beadchips were placed into a slide rack and submerged in a staining dish containing 250mL Wash E1BC solution. The slide rack holding all beadchips was then transferred to a staining dish containing 55ºC preheated High Temp Wash Buffer, and incubated for 10 min at 55ºC in an oven. After this incubation, the slide rack was transferred to a staining dish containing fresh Wash E1BC solution. This was briefly agitated using the slide rack, then placed on an orbital shaker at the highest possible setting that did not cause the solution to splash from the dish. The wash procedure was then repeated using 250mL of 100% ethanol, and again placed on an orbital shaker for 10 min. The wash procedure was repeated again using fresh Wash E1BC solution and placed on an orbital shaker for 2 min.

Blocking of the beadchips was performed by placing them face-up in 4mL of Block E1 buffer in a plastic wash tray, and rocked at medium speed for 10 min at room temperature. Staining was performed by placing the beadchips face-up in a fresh wash tray with 2mL Block E1 buffer and Streptavadin-Cy3 on a rocker for 10 min at medium speed at room temperature. The final wash step was involved placing beadchips in a slide rack in a staining dish containing 250mL of fresh Wash E1BC solution. This was briefly agitated and then placed on a orbital shaker at medium speed for 5 min. Beadchips held in a slide rack were placed in a centrifuge plate holder with paper towels and centrifuged at 275 x *g* at room temp for four min to completely dry before scanning was performed.

8.2.3.11 - Illumina 8x1 BeadChip scanning

The beadchips were all scanned using the Illumina BeadArray Reader and Illumina BeadScan software version 3.

8.2.3.12 - Illumina BeadChip Array analysis

Data generated from the BeadArray Scanner were analysed using Illumina BeadStudio Software version 3.0. Values for each sample were subjected to cubic spline normalisation, and the t-test error model was applied to all samples. Data was then imported to GeneSpring Software version 7.3.1 (Agilent). Data was normalised to the 50th percentile on a 'per chip' basis to control for intensity variations across the arrays, and a second 'per gene' normalisation was used to control for the variation in detection efficiency between genes signals.

Data was then imported to Microsoft Excel, and according to expression level values the genes could then be classified as 'off' for non-detected expression (0.01), 'lowly expressed'(0.011-0.499), 'normally expressed' (0.5-1.499) or highly expressed' ($\geq$1.5) for each time point. This was due to a value of 1 generated by GeneSpring to be representative of the median level of gene expression. By graphing genes in order of increasing expression, this classification appeared to accurately describe the level and pattern of gene expression. Genes were then further categorised according to the definitions below in Table 8.2.8 by taking into consideration the four assessed time points.

Table 8.2.8 – Definitions of gene categories. Based on these descriptions, all genes were classified in this way to enable comparison of genes between cell lines.

Category	Definition
Continually expressed	Normal Expression or higher across all 4 time points.
Not expressed	Expression is not detected at any time point.
Long term reactivation	Expression not detected in untreated cells, followed by normal or high expression in the next three time points
Short term reactivation	Expression was not detected in untreated cells, followed by high or normal expression level after drug treatment (d0), any level of expression at d4, and <100 fold expression (compared to treated) at the 10th day of drug free growth.
Permanently up-regulated	Expression was low in untreated cells, and was increased to normal or high after drug treatment and remained there in the following 10 days of drug free growth.
Temporarily up-regulated	Expression was low in untreated cells and was increased to normal or high after drug treatment (d0) and at d4, but returned to low expression at the 10th day (d10) of drug free growth.
Varied	Expression pattern does not fit into other categories.

Genes associated with CpG islands were of interest for further interrogation as they are likely to be controlled by epigenetic mechanisms. To determine which genes are associated with CpG islands, the University of California Santa Cruz (UCSC) Table Browser (available http://genome.ucsc.edu/cgi-bin/hgTables) was then utilised. Briefly, a 'custom track' of human genes associated with a CpG island was created, and this list was intersected with the entire list of gene names

generated from the Illumina Arrays. The output file describes the genes that are deemed to be related to a CpG island.

A list of genes which were classified as behaving differently after 5-aza-dC treatment (eg ‘Always on’ and ‘temporarily re-expressed) within different cell lines was then created for those genes with a CpG island. The purpose was to identify genes which behaved differently in different cell line with the aim of identifying methylation or histone modification differences which may contribute to this pattern of expression.

The SW480 and HCT116 cell lines were chosen for further investigations as these cell lines were identified to show differently behaving genes following 5-aza-dC treatment, recovery and re-methylation that were deemed suitable for our study. Both cell lines are originally taken from the colon, with the HCT116 cell line representing a carcinoma cell line of epithelial origin whilst the SW480 cells are listed as an adenocarcinoma cell line or glandular origin. By choosing two distinct forms of cells we avoid using cell lines which are too closely related and may share some common genetic alterations which may affect the epigenetic control of the genes of interest.

8.2.4 - Bisulfite Sequencing PCRs

8.2.4.1 - CpG Island Identification

Genes were chosen for Bisulfite Sequencing and Chromatin Immunoprecipitation analysis on the basis of the information generated from the Illumina expression array analysis. CpG islands were predicted with Methprimer software (Available http://www.urogene.org/methprimer/index1.html) [188] using approximately 4000bp of sequence surrounding the transcription start site of the gene of interest. Criteria for CpG island prediction were a 200bp minimum island size, scanning the sequence in 1bp steps, with an observed / expected CpG ratio of 0.6, and a GC percentage of 50%.

8.2.4.2 - Bisulfite Conversion

To determine the methylation status of individual cytosines, bisulfite conversion and sequencing was employed. This method involves incubating DNA in a high concentration of Sodium Bisulfite at a high temperature and low pH. These conditions are harsh and lead to a great deal of DNA degradation. However, it is in this environment where un-methylated cytosines are deaminated to uracil, whilst methylated cytosines are protected. The DNA is then desulphonated and purified using a column based method. After treatment, PCR and DNA sequencing is performed and a methylated cytosine will appear as a cytosine, whilst un-methylated cytosines that are converted to uracil will appear as a thymine residue.

Conversion was performed using the Qiagen EpiTect Bisulfite Conversion kit. DNA used in bisulphite conversion was treated with RNAse and phenol/chloroform purified as described above. 2µg of DNA in a volume of 20µL was added to 85µL of dissolved Bisulfite Mix and 35µL of DNA Protect buffer. Samples were mixed by pipetting and incubated in a thermal cycler under the following conditions as described in Table 8.2.9. Following conversion samples were mixed with 560µL of Buffer BL and bound to an Epitect Spin Column and spun at 17,000 x *g* for 60s. Samples were washed with 500µL wash buffer and centrifuged to wash the converted DNA. The supernatant was discarded and 500µL of Buffer BD was added and incubated for 15 min at room temperature to desulfonate the samples. Samples were then centrifuged at 17,000 x *g* for 60s, washed with Buffer BW and again centrifuged at 17,000 x g for 60s. Bisulfite converted DNA samples were eluted with the addition of 30uL EB buffer and centrifugation at 17,000 x *g* for 60s. 20µL of converted DNA was frozen at -20°C whilst the remaining 10µL was diluted 1:3 and used for PCR within 72 h.

Table 8.2.9 - Bisulfite Conversion Thermal Cycler Conditions

Step	Time	Temperature
Denaturation	5 min	99°C
Incubation	25 min	60°C
Denaturation	5 min	99°C
Incubation	1h 25 min	60°C

Denaturation	5 min	99°C
Incubation	2h 55 min	60°C
Hold	infinite	20°C

8.2.4.3 - Bisulfite Converted Polymerase Chain Reactions

Primers were designed to amplify a section of the CpG islands associated with either or both of the transcription start site (TSS) and translation start site. The conditions and volumes are listed below in table 8.2.10, however the thermal cycler conditions were very distinct and were dependant on the melting temperature of the primers in the reaction.

Table 8.2.10 - Components of single PCR reaction performed on bisulfite converted DNA

Component	Volume (µL)
water for injection	14.9
10X PCR buffer	2.5
dNTPS (2.5mM)	2.5
Forward / Reverse Primer (20uM)	1.25
$MgCl_2$ (50mM)	0.5
Taq Polymerase (5U/µl)	0.1
Template DNA (1:3 dilution)	2

A volume of 5µl of PCR reactions were then loaded onto a 1.5% (w/v) agarose gel with ethidium bromide and run at 90V for 40 min. The gel was viewed under ultra-violet light to determine if the PCR reactions were successful. Successfully amplified products were then purified using the Ampure method described previously and then used in a sequencing reaction.

8.2.4.4 - DNA Sequencing Analysis

Purified DNA was used in a sequencing reaction as outlined below in Table 8.2.11. A mastermix containing the components below was prepared and then the purified DNA was added. The volume of purified DNA in each reaction (*X)* was dependent upon the size of the fragment to be sequenced.

Table 8.2.11 - Components of a single sequencing reaction.

Component	Volume
5X Big Dye Buffer	3µl
Ready Reaction Premix	2µl
Primer (1µM)	4µl
Water For Injection	11-*x (µL)*
Purified DNA template	*x (µL)*

The sequencing reaction took place in a thermal cycler PCR instrument using the conditions described in Table 8.2.12 below. The 45-50ºC temperature was dependant on the melting temperature of the primers, and in each case the temperature used was 1ºC lower than that of the primer, or 50ºC if the melting temperature was higher.

Table 8.2.12 - Thermal cycler conditions used to perform a sequencing reaction.

Temperature	Time	Cycles
95°C	5 s	
45-50°C	5 s	x30
60°C	5 min	
4°C	Infinite	1

Following the sequencing reaction, the reactions were purified with CleanSeq (AgenCourt BioScience) which is similar to the Ampure method. 10µL of sequencing reaction was mixed with 18µL of CleanSeq magnetic beads, mixed and incubated at room temperature for 5 min. After this period, the plate was transferred to a magnetic plate holder where the magnetic beads attached to the DNA fragments were attracted to the magnet. Liquid was then aspirated with a pipette and discarded. The beads were then washed once with 85% ethanol for seconds. The ethanol was then removed and beads were allowed to air dry for 5 min before elution with 40µL of 0.1mM EDTA. After 5 min incubation, 20µl of the purified sequencing reaction was transferred to a 96 well plate. This plate was centrifuged, sealed and loaded into an ABI 3730 DNA Genetic Analyser for analysis.

Data files were then used in ABI Seq Scanner software version 1.0 where individual peak heights of cytosine and thymine at CpG sites were quantified using the Y-coordinate details. Samples were sequenced at least twice, and using the formula below, the value for each CpG was calculated and averaged then graphed against the CpG position for each gene and time point.

$$\text{Percentage methylcytosine} = \frac{\text{Height of cytosine peak} \times 100}{\text{Height of cytosine peak + height of thymine peak}}$$

8.2.5 - ChIP series of experiments

The ChIP assay was performed on cells at the time points of untreated, d0, d4, and d10 to assess the interactions between DNA methylation and histone protein modifications with regard to the expression of the gene. Cells were grown using the same procedure described in Chapter 8.2.1 with the exception that DNA, RNA and chromatin were collected in untreated, drug treated (d0), Day 4 (d4) and Day 10 (d10) of drug free growth, rather than each two days.

8.2.5.1 - Crosslinking of proteins and cell lysis

Cells were grown in a 100mm culture flask in 10ml of media. Formaldehyde was added to the cell culture media to a final concentration of 1% for 10 min at room temperature to form crosslinks between protein and DNA. Unreacted formaldehyde was quenched with the addition of 1.4mL of 1M glycine for a further 10 min at room temperature. All liquid media was removed and the cells were washed twice with ice-cold PBS containing 20µL protease inhibitors and incubated on ice for two min. Cell Lysis buffer (1mL containing 20µL protease inhibitors) (10mM HEPES, pH 7.9, 0.5% IGEPAL-CA630, 1.5mM MgCl2, 10mM KCl) was added to the culture dish and cells were scraped into a 2mL screw cap tube. To assist with cell lysis, the cells were aspirated three times with an 18g needle. Cells were incubated on ice for 15 min, and were vortexed at 5 min intervals. Cellular debris including the cell nucleus was pelleted by centrifugation at 17 x *g* for three min. The supernatant was discarded and cell pellets were resuspended in 800µL Nuclear Lysis Buffer (1% SDS, 10mM EDTA, 50mM Tris, pH 8.1 containing 20µL of protease inhibitors), and kept on ice. Aliquots containing 1 x 10^7 cells in 400µL were transferred to separate tubes, and a small amount of glass beads were added to each tube to enhance sonication.

8.2.5.2 - Shearing of chromatin

Sonication was performed with a Branson-250 sonifier to produce DNA fragments ranging in size from 100-700bp. Tubes containing 1x10^7 lysed cells were held in a saturated solution of -20ºC NaCl and ice during sonication. Cell lysates were subjected to 8 x 30 s cycles of sonication at 60% duty cycle, and

were rested for 30 s in saturated NaCl between cycles to keep the samples cool. Optimisation experiments revealed this would generate DNA fragments in the 100-700bp range when used in conjunction with the above cross-linking conditions.

8.2.5.3 - Pre-clearing and Immunoprecipitation

Sonicated cell lysates were centrifuged at 8000 x *g* for 5 min at 4ºC to pellet undissolved cell debris. Supernatants of duplicate tubes were then combined and gently mixed by pipetting. Aliquots representative of approximately two million cells each were then removed into separate 1.5mL DNA Lo-Bind tubes (Eppendorf) for each immunoprecipitation One extra aliquot was taken for the 'Input' sample, to serve as a reference for the amount of starting material at stored at 4ºC until purification. Samples for immunoprecipitation were made up to 500µL with Buffer 3 (1.124% Triton-X 100, 0.112% Sodium Deoxycholate, 1mM EDTA, 0.5617mM EDTA, 169mM NaCl, 10mM Tris-HCl, pH 8.1) to dilute the SDS. Pre-clearing was performed by the addition of 2µL of 1ug/mL Sonicated Salmon Sperm DNA (Sigma) and 40µL of a 1:1 mixture of Protein A Agarose beads (Biolab) and TE. The samples were placed on a rotating platform at 4ºC for at least 2 h to allow for non-specific binding of chromatin to the Protein A Agarose beads. After 2 h the samples were centrifuged at 8000 x *g* for 1 min to pellet the agarose beads. The supernatant was then removed to a new 1.5mL LoBind tube ensuring no beads were removed. The appropriate antibody was then added to each supernatant according to Table 8.2.13 and returned to the rotating platform at 4ºC for at least 16 h. Optimisation experiments revealed this amount of antibody is appropriate for maximum recovery of the desired fraction from two million cells.

Table 8.2.13 - Volume of antibody used in a single immunoprecipitation reaction.

Antibody	Volume (µL)
α-acteyl H3	5
αH3K4me3	5
αH3K9me3	4
αH3K27me3	4
Rabbit IgG	5

8.2.5.4 - Capturing of Immunoprecipitation complexes, washing and elution

Chromatin-antibody complexes were captured by the addition of 2uL 1ug/ml sonicated salmon sperm DNA and 40µl of a 1:1 mixture of Protein A Agarose beads and TE and were again returned to the rotating platform at 4ºC for at least 90 min. These samples were then centrifuged at 8000 x *g* for 1 min at 4ºC and the supernatant was discarded. The antibody-chromatin complexes were then bound to the agarose beads and were washed several times according to the following procedure to remove non-specific binding.

i) 1mL TSE I buffer (10mM Tris-HCl, 1mM EDTA) added, inverted several times and incubated on ice for 10 min. Tubes were then inverted every few minutes to ensure adequate washing.
ii) Samples centrifuged at 8000 x *g* for 1 min at room temperature.
iii) Supernatant discarded whilst not disturbing the pellet of beads.
iv) Repeat steps i) to iii) with TSE II (0.1% SDS, 1% Triton-X 100, 2mM EDTA, 20mM Tris-HCl, 500mM NaCl) buffer and LiCl buffer (2mM LiCl).
v) Resuspend pellet in TE and centrifuge for 1 min at 8000 x *g*. Discard supernatant.
vi) Repeat step v)
vii) Resuspend in TE and transfer beads and TE solution to a new LoBind tube to minimise contamination with non-specific binding of sample attached to the original tube.
viii) Repeat centrifugation and discard supernatant.

The chromatin-DNA complexes were then eluted from the agarose beads with the addition of 150µL 55ºC preheated elution buffer and placed in a 55ºC hot block for 10 min, with gentle inversion every ~3 min. Agarose beads with collected with centrifugation for 1 min at 8000 x *g* at room temperate and supernatant containing eluted chromatin-DNA complexes were removed to a new 1.5mL tube. Further elution of immune complexes was achieved with

another two washes cycles with 100µl elution buffer, centrifugation and combining of all corresponding supernatants.

8.2.5.5 - Purification of DNA

Crosslinks of DNA and chromatin from Immuno-precipitated samples and the Input samples were reversed with the addition of 20µL Proteinase K 20mg/mL (Promega) and incubated at 62º for at 3 h with shaking. A final denaturation was performed by heating the samples to 70ºC for 10 min.

Final purification was performed with a Qiagen PCR clean up kit following manufacturer's instructions. Briefly, samples are mixed with five volumes of Buffer PBI to facilitate binding to the membrane within a spin-column. Samples were centrifuged at 17,000 x *g* for 1 min and supernatant was discarded. Samples were washed twice with wash buffer, before elution in 30µL of elution buffer supplied with the kit.

8.2.5.6 - Quantification of Immunoprecipitated DNA using qPCR

DNA to be used in ChIP-PCRs was firstly quantified to ensure the same amount of DNA was available in each PCR. Input DNA fractions were diluted 1:400, while other fractions were diluted 1:4 or 1:20 for higher concentration samples. The DNA Quantitation System (Promega) was used to accurately determine the low quantities of DNA present in samples using light produced from an enzymatic reaction. A mastermix using the components listed below in Table 8.2.14 was prepared, and 18µL of this reaction was mixed with 2µL of DNA sample and incubated at 37ºC for 8-10 min. Samples were then immediately placed on ice until read using a TD 20/20 luminometer (Turner Designs). 15µL of sample was then mixed with 100µL of ENLITEN Reagent and immediately placed in the luminometer, which was set to have a 3 sec delay and a 15 sec read time. A standard curve was produced and graphed allowing the unknown sample concentrations to be calculated.

Table 8.2.14 - The components of a mastermix required in a single DNA quantitation

Component	Volume (µL)
DNA Quantitation Buffer	15.5
Sodium Pyrophosphate	0.5
NDPK Enzyme Solution	0.1
Water For Injection	0.9
T4 DNA Polymerase	1.0

8.2.5.7 - ChIP PCR reactions

The presence of particular histone modifications at specific genes was detected with real time PCR. Primers were designed to amplify a small region within the CpG island of the target gene of interest. A mastermix was prepared according to the volumes outlined in Table 8.2.15, along with 200ρg of immunoprecipitated DNA. Reactions were performed in triplicate using an ABI 7500 PCR machine using the conditions listed below in Table 8.2.16. C_T values were determined automatically by Sequence Detection Software version 1.4 (Applied Biosystems) and the values were expressed as a percentage of the input fraction, using the following equation;

$$\text{Percentage Input} = 2^{(\Delta Ct\ Target)-(\Delta Ct\ Input)}$$

Table 8.2.15 - Components of a mastermix required for a single ChIP-PCR reaction

Component	Volume (µL)
2X SYBR Green Mastermix	5
Forward / Reverse Primer (10uM)	0.5
Water For Injection	2
Template DNA	2

Table 8.2.16 - Typical PCR conditions used to perform ChIP-PCR and qPCR reactions. Some genes PCR's required a slightly hotter annealing/extension step denoted by the asterisk.

Temperature	Time	Cycles
50°C	2 min	1
95°C	10 min	1
95°C	15 s	
60°C *	1 min	x45

8.2.5.8 - Gene Expression Quantification

In order to quantify the expression of mRNA transcripts, extracted RNA was converted to cDNA by Reverse Transcription. Up to 5µg of total RNA was made up to 11µLwith sterile distilled water, with the addition of 1µL (250ng) random primer and 1µL dNTP mix (10mM each). This mixture was heated to 65ºC for 5 min and immediately placed on ice. After short centrifugation, 4µL 5X First-Strand buffer and 2µL 0.1M DTT was added, mixed gently and incubated at 42ºC for two min. Following incubation, 1µL (200U) of SuperScript II Reverse Transcriptase was added, then initially incubated for 10 min at 25ºC before a 50 min incubation at 42ºC. The reaction was inactivated with 15 min incubation at 70ºC. The synthesised cDNA was then diluted 1:10 with sterile Milli Q water prior to PCR.

8.2.5.9 - qPCR to determine gene expression

The expression of specific gene mRNA was quantitated by performing PCR on the cDNA of the samples. A mastermix was prepared using the components listed in Table 8.2.15. Reactions were performed in triplicate using an ABI 7500 PCR machine using the conditions listed in Table 8.2.16. C_T values were determined automatically by Sequence Detection Software version 1.4 (Applied Biosystems) and final calculations were expressed as fold differences using the $\Delta\Delta C_T$ method. This involves calculation of the average C_T value for the target gene and the house keeping gene, β-actin. The ΔC_T, $\Delta\Delta C_T$ and fold differences were calculated as follows.

ΔC_T = (Average C_T Target gene) – (Average C_T β-actin)

$\Delta\Delta C_T$ = (ΔC_T treated) – (ΔC_T untreated)

Fold Change in expression = $2^{-\Delta\Delta CT}$

Table 8.2.17 - The components of a mastermix required for a single qPCR reaction

Component	Volume (µL)
2X SYBR Green Mastermix	5
Forward / Reverse Primer (250nM)	2.5
Water For Injection	0.5
Template cDNA	2

8.2.6 – Methods Optimisation

Each of the experiments described in this thesis required optimisation to ensure the methodology was acceptable such that accurate interpretation of the biological observations could be made. A description of the optimisation of each experiment is described below.

8.2.6.1 – AvrII Enzymatic Digestion of PCR products

- Agarose gel electrophoresis of PCR products was performed to ensure the correct size fragment was amplified.
- A series of digestions were performed using varying concentration AvrII to identify the minimum amount of enzyme required to adequately digest a fixed volume of PCR product.

8.2.6.2 – Nuclease P1 Enzymatic Digestion of genomic DNA

- Varying concentrations of DNA were used with a constant volume of Nuclease P1 enzyme to determine the maximum amount of DNA that could be used to ensure a sufficient signal to noise ratio was obtained.
- HPLC flow rates were optimised to minimise run times and carry-over effects of previous sample injections.

8.2.6.2 – Drug treatment for cell lines

- Cell lines were treated with 5-aza-dC at 5µM, 10µM, 15µM and 20µM concentrations for 72 hours. DNA was extracted and analysed by HPLC to determine the lowest concentration that would induce maximal demethylation.

8.2.6.3 – Quantitative Polymerase Chain Reaction

- For each qPCR reaction (expression or ChIP style) the annealing/extension termperature was optimised to ensure efficient amplification, according to manufacturers instructions. A dilution series of cDNA or sheared chromatin was prepared using a 1:10 dilution to create five test samples. Standard curves were generated for each PCR, plotting Cycle Threshold value against the log of the input. A PCR was deemed efficient is the 'slope' was -3.32 $\pm$0.25 and the R^2 replicates were $\geq$0.95.

8.2.6.4 – ChIP optimisation

- The sonication of each cell line was optimised to ensure the fragment sizes were largely within the 100-500bp range. The duty cycle was kept constant at 60%, as were the 30sec on/off intervals, whilst a range of cycle numbers was to determine which setting best met the size range of fragments produced.
- A titration was performed to determine the sufficient amount of antibody required to capture the maximum amount of targets. On a constant series of chromatin samples representative of an experimental sample, varying volumes of antibody were used to capture target DNA. Quantitative PCR was used to determine the lowest volume of antibody required to immuno-precipitate the maximum quantity of DNA.