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Experimental Approaches to the Study of Epigenomic Dysregulation in Ageing

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Abstract

In this review, we describe how normal ageing may involve the acquisition of epigenetic errors over time, akin to the accumulation of genetic mutations with ageing. We describe how such experiments are currently performed, their limitations technically and analytically and their application to ageing research.

The Epigenome

The epigenome is loosely defined as the genome-wide collection of epigenetic regulatory processes acting on the genome. Epigenetic regulation is literally a description of those mechanisms that act ‘upon’ the genome, and as such could encompass all transcription regulatory processes, but is usually limited to those that can be inherited from parent to daughter cell, or from generation to generation. Such regulatory processes play an important role in cellular differentiation and the maintenance of a cell type within a given lineage. Stem cell differentiation inherently involves epigenetic changes (Bernstein et al., 2006a), with consequent reprogramming of transcriptional profiles.

Epigenetic Regulatory Processes

Cytosine methylation

A number of epigenetic regulatory processes have been described. Of these, cytosine methylation exhibits the best evidence for heritability from parent to daughter cells. In mammals, cytosine methylation is localized almost exclusively to CG dinucleotides (~3-8% of all cytosines in the genome) and occurs symmetrically on both strands of DNA (Bird, 1986). Following DNA synthesis, two new hemimethylated strands of DNA each acquire symmetric methylation, enzymatically copied from the methylated to the unmethylated strand by DNA methyltransferase 1 (Dnmt1), with S-adenosylmethionine as the methyl donor (Hermann et al., 2004). An additional class of DNA methyltransferases is responsible for *de*

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*nov*o methylation. These include Dnmt3a and Dnmt3b, both of which are robustly expressed in undifferentiated pluripotent embryonic stem cells and downregulated upon cell differentiation (Okano et al., 1999; Okano et al., 1998). Dnmt3a and Dnmt3b exhibit nonoverlapping functions in development, with Dnmt3b specifically required for methylation of centromeric satellite DNA repeats (Okano et al., 1999). Dnmt3a, on the other hand, is implicated in a number of functions including nucleolar inactivation in resting and growth-arrested cells (Galetzka et al., 2006). Additionally, Dnmt3a may act in concert with its regulatory binding partner Dnmt3L (Suetake et al., 2004), a DNA methyltransferase-like protein that plays a central role in the establishment of maternal genomic imprinting but does not have catalytic activity on its own (Hata et al., 2002; Nimura et al., 2006). Dnmt3L serves a targeting role by binding unmethylated histone tails and by activating *de novo* DNA methyltransferase activity in a tetrameric complex with Dnmt3a (Jia et al., 2007). Interestingly, the *DNMT3A* gene encodes two active isoforms with strikingly different tissue expression and subcellular localization patterns: Dnmt3a is concentrated on heterochromatin and Dnmt3a2 is associated with euchromatin (Chen et al., 2002).

Methyl groups are typically attached at the 5-carbon position of cytosine and protrude into the major groove of the DNA where they are accessible for recognition by methylation-sensitive transcription factors (*e.g.* CTCF (Bell and Felsenfeld, 2000; Hark et al., 2000)) and methyl-binding proteins that are associated with gene silencing (Jorgensen and Bird, 2002; Sansom et al., 2007). Some methyl-binding proteins may even interact with multiple adjacent methylated cytosines (Filion et al., 2006). Moreover, these methylation-sensitive, DNA bound factors may recruit additional complexes (*e.g.* histone deacetylases) for modulation of chromatin state and transcriptional activity (Jones et al., 1998; Nan et al., 1998).

The MBD (methyl-CpG-binding domain) protein family is composed of five known members (MeCP2, MBD1, MBD2, MBD3, and MBD4), proteins that recruit histone modifying and chromatin remodeling complexes to methylated sites. In cancer, MBD proteins have been found to occupy hypermethylated promoters of tumor suppressor genes (Lopez-Serra et al., 2006) and miRNAs (Lujambio et al., 2007), correlating with silencing of expression at these loci. Moreover, knockdown of MBD expression using targeted RNAi was shown to release the transcriptional repression of many hypermethylated genes, without affecting their DNA methylation status (Lopez-Serra et al., 2008).

MeCP2 mediates gene repression by its association with a number of chromatin remodeling complexes. The transcriptional repression domain (TRD) of MeCP2 interacts directly with Sin3A and an associated complex of histone deacetylases (*i.e.* HDAC1 and HDAC2) (Nan et al., 1998). It is also known that MeCP2 can recruit histone H3 methyltransferase to regions of DNA methylation; however, the identity of this complex is still unknown (Fuks et al., 2003a). Indeed, MeCP2 is likely to interact with a number of other cofactors to mediate transcriptional repression in response to DNA methylation (Klose and Bird, 2004). Moreover, its function is required for proper development, with mutations of either the MeCP2 MBD or TRD domains causing Rett syndrome (Amir et al., 1999).

MBD1 is also able to bind DNA in a methylation-dependent manner and function as a transcriptional repressor. MBD1 consists of multiple isoforms, each of which possesses a different affinity for methylated DNA (Fujita et al., 2000). Interestingly, isoform MBD1a, containing a CXXC3 domain, specifically binds unmethylated CG dinucleotides, while retaining its transcriptionally repressive function (Jorgensen et al., 2004). MBD1 repressive activity is mediated by SETDB1 histone methyltransferase recruitment (Sarraf and Stancheva, 2004) but MBD1 has also been shown to interact with additional histone methyltransferases (*e.g.* Suv39h) (Fujita et al., 2003).

Repressive activity of MBD2 and MBD3 are both mediated by an ATP-dependent chromatin remodeling complex, consisting of at least 10 major proteins including multiple nucleosome remodeling and histone deacetylase (NuRD) components (Feng and Zhang, 2001; Le Guezennec et al., 2006). However, the MBD2 and MBD3 complexes have type-specific components and distinct functions (Hendrich et al., 2001). The MBD2-containing form functions as MeCP1, a large complex that binds regions containing > 12 methylated CG dinucleotides, thus associating with loci of higher CG density (e.g. CpG islands) (Boyes and Bird, 1992). MBD2 is also involved in methylation-dependent gene silencing of *Xist*, playing an essential role in X chromosome inactivation (Barr et al., 2007). The MBD2 but not the MBD3 complex consists of additional effectors such as PRMT5, an arginine methyltransferase (Fabbri et al., 2002), and its cofactor MEP50 (Le Guezennec et al., 2006). On the other hand, both MBD2 and MBD3 complexes contain histone deacetylases HDAC1 and HDAC2. Interestingly, MBD3 contains a disrupted methyl-CpG-binding domain and is unable to recognize methylated DNA directly (Hendrich and Bird, 1998). Instead, it is proposed that chromatin remodeling is established by MBD in densely methylated regions, with subsequent recognition of chromatin marks by the MBD3-containing Mi-2/NuRD complex (Le Guezennec et al., 2006).

In addition to the MBD family of proteins, there are a variety of zinc finger proteins that have the ability to recognize and bind methylated DNA as well as to recruit additional epigenetic regulators. Kaiso, a member of the BTB/POZ zinc finger domain family, binds methylated DNA in the context of a 5'-CGCG-3' consensus sequence containing two consecutively methylated CGs (Prokhortchouk et al., 2001). Kaiso-dependent transcriptional repression is mediated by the N-CoR corepressor complex and histone deacetylase (HDAC3) activity (Yoon et al., 2003). Two other known zinc finger proteins similar to Kaiso, ZBTB4 and ZBTB38, are also able to bind methylated CG dinucleotides and inhibit local gene expression (Filion et al., 2006).

Post-translational modifications of histone proteins

Chromatin, a packaged form of DNA within the cell, is composed of a string of nucleosomal "beads" (Olins and Olins, 1974; Oudet et al., 1975), each of which consists of 147 bp of DNA wrapped around an octamer of four core histone proteins (H2A, H2B, H3, and H4) (Richmond and Davey, 2003). Linker histone H1 sits at the junction of this 'nucleosomal' DNA and the internucleosomal DNA sequence (Noll and Kornberg, 1977; Whitlock and Simpson, 1976). Nucleosomes are highly stable protein-DNA complexes, formed by fourteen different sites of interaction between the core histones and the DNA (Luger et al., 1997). Each of the core histones has a highly globular structure, with the exception of their N-terminal tails which protrude from the nucleosomal unit and are accessible to enzymes in the surrounding environment (Luger et al., 1997; Whitlock and Simpson, 1977).

Chromatin can exist in the cell in multiple different states. When a region of DNA is actively being transcribed, it exists as euchromatin, loosely packaged around nucleosomes in the form of "beads-on-a-string" (Oudet et al., 1975; Thoma and Koller, 1977). When the DNA is poised for transcription, but not actively serving as a template for mRNA production, it remains as euchromatin, but is packaged into a supercoiled 30nm fiber (Dorigo et al., 2004; Mozziconacci and Victor, 2003; Schalch et al., 2005), whereas heterochromatin is associated with further supercoiling and packaging of the 30nm fiber into higher-density structures along with scaffolding proteins (e.g. NuMA) (Barboro et al., 2003). During mitosis, chromatin ceases to function as accessible genetic material and transcription stops as the DNA is packaged into extraordinarily dense structures called chromatids.

Specific amino acids on the histones can be subject to a wide variety of covalent but reversible modifications, which can act alone or in concert to generate enormous complexity. At least

eight different classes of histone tail modifications (methylation, acetylation, ubiquitylation, ADP-ribosylation, sumoylation, deimination, phosphorylation, and proline isomerization) occur at over 60 different amino acid residues including multiple lysines, arginines, serines, and threonines (Kouzarides, 2007). Moreover, some of these modifications can occur in multiple forms (e.g. mono-, di-, or trimethyl lysine) (DeLange et al., 1972), whereas some modifications (e.g. acetylation) carry a negative charge which can neutralize the basic charge of lysines, allowing the adjacent DNA to loosen from the nucleosome (Anderson et al., 2001; Choi et al., 2008). These modifications can mediate direct effects on chromatin structure and can recruit accessory proteins to manipulate chromatin packaging and transcriptional control. Histone tail acetylation is predominantly associated with activation of transcription (Hebbes et al., 1988), and tends to be enriched at specific sites in the promoters and 5' ends of coding regions (Liu et al., 2005; Pokholok et al., 2005). In addition, histone H4 acetylation at lysine position 16 (H4K16ac) can also prevent compact 30 nm fiber formation and lead to chromatin decondensation (Shogren-Knaak et al., 2006). Conversely, histone tail deacetylation correlates with transcriptional repression. Perhaps unsurprisingly, H4 acetylation is largely absent from the inactive X chromosome (Jeppesen and Turner, 1993).

Histone methylation can occur on either lysine or arginine residues, and can be either activating or repressive for expression. Methylation of three lysine residues (H3K4, H3K36, and H3K79) is associated with transcriptional activation and elongation (Joshi and Struhl, 2005). Interestingly, H3K36me, localized toward the 3' end of active genes, is implicated in suppressing inappropriate transcriptional initiation from cryptic start sites within the coding region (Carrozza et al., 2005). Loss of methylation at H3K4 conversely acts to repress transcription (Shi et al., 2004). Three other methylated lysine residues (H3K9, H3K27 and H4K20) are generally implicated in silencing active euchromatin. H3K27me3 is specifically associated with HOX gene repression as well as X chromosome inactivation; however, it is also enriched along with H3K4me3 at bivalent domains in pluripotent stem cells that are poised to be turned on or off (Bernstein et al., 2006b; Mikkelsen et al., 2007). H3K9me3, repressive at gene promoters, is paradoxically associated with coding regions of active genes (Vakoc et al., 2005). H4K20 methylation has been shown to associate with sites of DNA repair (Sanders et al., 2004). Like lysine methylation, arginine methylation can be either activating or repressive, depending on the context. At the estrogen-regulated pS2 promoter, arginine methylation appears to be established in a cyclical manner (Metivier et al., 2003).

Numerous histone modifications may coexist in close proximity, or they may be mutually exclusive, either situation potentially affecting function. For instance, individual lysine residues cannot sustain more than a single class of posttranslational modification, and modifications at multiple lysines may be mutually exclusive (Fischle et al., 2003; Garcia et al., 2007). Furthermore, the recognition of an individual modification may be affected by the presence or absence of a neighboring modification. Heterochromatin protein 1 (HP1) recognition of H3K9 methylation is an example of this, as its binding is negatively dependent upon the phosphorylation state of the adjacent residue, H3 serine 10 (Fischle et al., 2005). Conversely, H3 serine 10 phosphorylation can enhance GCN5 acetyltransferase binding to deacetylated H3K14, and leads to increased H3K14 acetylation (Clements et al., 2003). Additionally, cross-talk between multiple histone modifications may also occur, as ubiquitination of H2B is required for methylation of H3K4 (Sun and Allis, 2002), whereas modifications such as proline isomerization can completely disrupt recognition of other modifications on the same histone tail (Nelson et al., 2006).

Taken together, histone modifications appear to generate a complex code that can function in the establishment of a global chromatin environment, with far-reaching consequences for most if not all chromatin-templated processes such as transcription (Strahl and Allis, 2000).

ATP-dependent chromatin remodeling complexes

In addition to the enzymes mediating the histone modifications described above, ATP-dependent chromatin remodeling complexes can be recruited to the genome and can affect nucleosomal DNA accessibility to transcription factors (Narlikar et al., 2002). While ATP-dependent chromatin remodeling complexes can bind to DNA nonspecifically, multiple studies demonstrate that chromatin remodeling is a site-specific process, regulated at the single-gene level both spatially and temporally (Holstege et al., 1998; Sudarsanam et al., 2000). Chromatin remodeling complexes are able both to activate (Neely et al., 1999) and to repress (Goldmark et al., 2000) local transcription. Divided into multiple classes in mammals (SWI/SNF/Brm, ISWI, Mi2/NuRD, and the split ATP-ases INO80 and SWR1), ATP-dependent chromatin remodeling complexes can affect nucleosomes through at least four different mechanisms (Cairns, 2007). Remodelers can displace the histone octamer by sliding or moving it to a new position, thus exposing DNA in its original place (Hamiche et al., 1999; Langst et al., 1999; Whitehouse et al., 1999). Chromatin remodeling can also occur by complete removal of a histone octamer (Boeger et al., 2004; Lorch et al., 1999). Alternatively, active removal of histone H2A and H2B dimers can destabilize the remaining nucleosome H3/H4 tetramer, thus loosening and exposing the apposed DNA (Yang et al., 2007). Finally, histone variants can be exchanged for resident components of the nucleosomal octamer (e.g. H2A.Z/H2B exchange for H2A/H2B) (Mizuguchi et al., 2004). Most ATP-dependent chromatin remodeling complexes possess the ability to translocate nucleosomes although some (e.g. ISWI) maintain even spacing between histone octamers while others (e.g. SWI/SNF) do not (Ito et al., 1997; Owen-Hughes et al., 1996). SWI/SNF and ISWI complexes are also distinguished by their differential abilities to eject both histone octamers and H2A/H2B dimers (Bruno et al., 2003; Lorch et al., 2006; Lorch et al., 1999; Yang et al., 2007). Histone variant exchange on the other hand is limited to the opposing functions of SWR1 and INO80 (Mizuguchi et al., 2004; Papamichos-Chronakis et al., 2006).

Chromatin remodeling complexes can alter transcriptional status in a locus-specific manner. Not only are they able to stimulate binding of gene-specific activators and components of the transcriptional preinitiation complex (e.g. TATA binding protein) (Burns and Peterson, 1997; Cote et al., 1994; Imbalzano et al., 1994), ATP-dependent chromatin remodeling complexes are able to increase the overall rate of transcription from nucleosomal templates (Neely et al., 1999). Moreover, each remodeling complex has a different ability to act at certain sites (Hassan et al., 2001; Lemon et al., 2001). In highly condensed chromatin, widespread during late mitosis, ATP-dependent remodeling complexes are essential for gene expression, whereas they may or may not be required at the same loci during interphase when chromatin at these loci may be more generally accessible (Krebs et al., 2000). The maintenance of transcriptional state at specific genes (e.g. GAL1) from parent to daughter cells through cell division is also critically dependent on SWI/SNF activity (Kundu et al., 2007).

Indirectly, ATP-dependent chromatin remodeling complexes can coordinately interact with histone modifying enzymes: histone acetylation can enhance ATP-dependent chromatin remodeling, just as remodeled chromatin can serve as a higher-affinity target for histone modifying enzyme (e.g. deacetylase) activity (DiRenzo et al., 2000; Guschin et al., 2000). Moreover, direct interactions between ATP-dependent chromatin remodeling complexes and histone tail modifying enzymes provide an additional layer of transcriptional regulatory control (Pollard and Peterson, 1997; Roberts and Winston, 1997). For instance, chromatin remodeling complexes can, along with certain histone acetyltransferases, cooperatively and selectively alter chromatin structure to activate site-specific meiotic recombination at specific points during the cell cycle (Yamada et al., 2004). Additionally, transcription of a single metabolic gene (i.e. SUC2) is dependent upon the cooperative interaction of the SWI/SNF complex with two different HATs, Gcn5p and Esa1p (Geng and Laurent, 2004). Similarly, protein arginine

methyltransferases (e.g. Prmt5 and Carm1/Prmt4) have also been shown to be required for ATP-dependent chromatin remodeling and locus-specific gene activation during tissue differentiation (Dacwag et al., 2009; Dacwag et al., 2007).

Interactions of Chromatin Organization, Histone Modifications and DNA Methylation

As discussed previously (see above), methyl binding domain (MBD) containing proteins can serve as adapters between DNA methylation status and histone-modifying and chromatin-remodeling complexes. However, the DNA methyltransferases can also interact with chromatin constituents and modifying proteins. For instance, Dnmt1, Dnmt3a, and Dnmt3b have all been shown to interact and cooperate functionally with heterochromatin protein 1 (HP1[?], [?], and [?]) (Fuks et al., 2003b; Smallwood et al., 2007), which itself recognizes modified histone tails (e.g. methylated H3K9 (Lachner et al., 2001)) and, as its name suggests, helps to form heterochromatin. HP1, with highest *in vivo* affinity for the *de novo* methyltransferase Dnmt3b, can therefore recruit this enzymatic activity to sites of H3K9 methylation (e.g. pericentromeric heterochromatin) (Lehnertz et al., 2003). Moreover, the isoforms of HP1 preferentially associate with different parts of the genome during different phases of the cell cycle (Hayakawa et al., 2003), therefore HP1 may direct Dnmt activity with changing specificity throughout the cell cycle. In addition to the methyltransferases, HP1 also associates with at least one MBD protein (i.e. MeCP2) (Agarwal et al., 2007), suggesting that H3K9 methylation can recruit DNA methylation, but that DNA methylation in turn can recruit heterochromatin proteins.

Histone deacetylases HDAC1 and HDAC2 have been shown to directly interact with DNA methyltransferase Dnmt1 (Robertson et al., 2000; Rountree et al., 2000). Moreover, inhibition of histone deacetylases with trichostatin A (TSA), has the additional effect of altering locus-specific DNA methylation, implying a functional link between HDAC and targeted Dnmt activity (Selker, 1998). Additionally, histone deacetylase activity in the context of NuRD ATP-dependent nucleosome remodeling complex can be directed by MBD2, allowing the recruitment of gene silencing machinery to methylated DNA (Zhang et al., 1999). Taken together, these studies suggest that DNA methylation can recruit histone deacetylase activity and, conversely, that HDACs are able to direct locus-specific DNA methylation.

Histone methylation and the histone methyltransferases have also been connected with DNA methyltransferase activity, on both a local and global level. Globally, the histone methyltransferase (e.g. SET7) and histone demethylase (e.g. LSD1) machinery is capable of specifically methylating and demethylating the Dnmt1 enzyme, respectively (Esteve et al., 2009; Wang et al., 2009). These modifications can affect Dnmt1 stability at different stages of the cell cycle and correlate with decreased and increased DNA methyltransferase activity, respectively. Additionally, *de novo* methyltransferase Dnmt3a was recently shown to recognize and bind to histone H4 arginine methylation, with loss of PRMT5 function leading to reduced Dnmt3a binding, loss of local DNA methylation and subsequent gene activation (Zhao et al., 2009). Dnmt3a can also recognize unmethylated H3K4 indirectly via Dnmt3L, further linking *de novo* DNA methylation with histone tail modifications (Ooi et al., 2007).

A number of other diverse interactions link multiple epigenetic modifications. Constitutive centromere protein (CENP-C) can interact with Dnmt3b and therefore direct DNA methylation to centromeric and pericentromeric satellite repeats (Gopalakrishnan et al., 2009). CXXC finger protein 1 (Cfp1) is a known regulator of cytosine and histone methylation as well as methyltransferase activity, and it contains a number of functional domains that may mediate epigenetic cross-talk (Butler et al., 2009; Tate et al., 2009). Although the mechanism is currently unknown, the gene silencing machinery may also recruit *de novo* DNA methylation

and progressive histone remodeling, as has been shown to occur for a nucleosome-free CpG island of the *CDKN2A* gene (Hinshelwood et al., 2009).

Recent discoveries suggest that RNA-based mechanisms may also play a role in coordinating the epigenome. Large non-coding RNAs such as *Air* are able to silence epigenetically the transcription of specific target genes (i.e. *Slc22a3*) through recruitment of repressive histone modifying activity, for example the H3K9 histone methyltransferase G9a (Nagano et al., 2008). RNAs, most notably *Xist*, also play multiple significant roles in establishing higher-order chromatin states (e.g. X chromosome inactivation) (Kanduri et al., 2009). Beyond these and a handful of other examples however, the role of RNAs in either targeting or modulating epigenetic marks and machinery remains unclear.

Tools to Study the Epigenome

There are a broad variety of tools available to study epigenetic marks throughout the genome as well as the factors that regulate them. Some tools are designed to target DNA methylation at single CG dinucleotides, at hundreds or thousands of base pairs at a time, or simultaneously throughout the entire genome, while other tools may only quantify global levels of cytosine methylation. Besides methylation-focused approaches, a number of tools also exist to interrogate histone modifications, DNA-associated proteins, and chromatin structure at the local or genome-wide levels. Each approach has its relative strengths and weaknesses, and each may be optimally suited to a different study depending on a number of factors including cost, quantitative resolution, data throughput, and DNA amount or quality. It should be noted, however, that despite the wide range of epigenetic regulators available for study, the focus has traditionally been almost exclusively on cytosine methylation, using the various approaches illustrated in Fig. 1. In large part this reflects the relative ease of analysis of this biochemical process, although the hope is that advances in technologies to study chromatin will make comparable studies of chromatin constituents equally as feasible.

1. Analysis of DNA Methylation

a. Methylation-dependent restriction enzyme-based approaches—A wide variety of restriction enzymes are known to recognize DNA in a methylation-dependent manner, cutting only if the DNA is unmethylated or, in less common cases, when the DNA is methylated. Pairs of such enzymes, where both recognize the same motif but each exhibits a different ability to digest the DNA depending upon methylation status, are termed isoschizomers. Many methylation-sensitive restriction enzymes are used in a variety of DNA methylation assays; however, the most commonly used of these restriction enzymes is HpaII, which recognizes and digests the unmethylated 5'-CCGG-3' motif. MspI, an isoschizomer of HpaII, recognizes and digests the same tetranucleotide motif irrespective of methylation status (Waalwijk and Flavell, 1978). HpaII and MspI are used, along with many other isoschizomer pairs and other methylation-sensitive enzymes, for both single locus and genome-wide analyses.

One of the earliest methods to test DNA methylation patterns genome-wide was restriction landmark genomic scanning (RLGS), which uses two-dimensional (2D) gel electrophoresis to compare methylation-sensitive digests from two different samples (Kawai et al., 1993). In RLGS, a rare-cutting methylation-sensitive restriction enzyme (e.g. NotI: 5'-GCGGCGCC-3') generates unique digestion profiles for each differentially methylated sample, which each show different 2D gel spot profiles. Current analytical advances allow rapid interpretation of methylation data from RLGS (Wimmer et al., 2002), and the accuracy of this technique has been proven, however it remains a technical challenge to perform and generates limited amounts of data (Ushijima, 2005).

PCR-based approaches were subsequently developed in order to target analyses to specific genes and sequences. The most straightforward assay involves a restriction digest followed by PCR amplification across the restriction site. If the methylation-sensitive enzyme cuts, PCR cannot amplify that locus thus providing a readout of methylation status at a given restriction site. Moreover, this approach (MSRE-PCR) can be multiplexed to assess many loci in parallel, demonstrated for as many as 53 promoter sites at the same time (Melnikov et al., 2005). Similarly, ligation-mediated PCR (LM-PCR) measures the ability of a methylation-sensitive enzyme to digest a site proximal to a gene-specific primer (Schmitz et al., 1997). An alternative technique, COMPARE-MS, combines methylation-sensitive restriction digests with precipitation of methylated DNA by affinity-based approaches (described below) followed by locus-specific PCR spanning a methylation-sensitive restriction site (Yegnasubramanian et al., 2006). The motivation for this combined approach is that pre-digested DNA might help to increase the specificity of methylated precipitates; however, this approach still remains subject to a number of limitations.

In recent years, microarray technologies have been adapted to the study of DNA methylation. The pioneering such technique, Differential Methylation Hybridization (DMH) relies on endonuclease digestion at non-CG sites (e.g. MseI, which cuts 5'-TTAA-3') followed by adapter ligation, methylation-sensitive digestion, enrichment of methylated sites by PCR, and comparative microarray hybridization using two labeled samples (Huang et al., 1999). Alternative approaches such as MCA (methylated CpG island amplification) (Toyota et al., 1999), AIMS (amplification of inter-methylated sites) (Frigola et al., 2002), and MTA (methylation target array) (Chen et al., 2003) also enrich the methylated fraction of the genome by using methylation-sensitive restriction enzymes to digest and therefore degrade unmethylated regions of DNA. In all cases, pairs of differentially methylated representations can be hybridized onto microarrays for comparative genome-wide analyses or onto metaphase chromosomes for visualization of regional variations in methylation. MSNP combines methylation-sensitive restriction analysis and LM-PCR with Affymetrix-based SNP arrays to measure methylation status near each SNP represented (Yuan et al., 2006). Two other approaches, MMAS (Ibrahim et al., 2006) and aPRIMES (Pfister et al., 2007), are based on the same technique and analytical platform, but differ in that they use comparative hybridization of two methylation-sensitive digests, one enriching for unmethylated DNA (McrBC digests methylated DNA (Sutherland et al., 1992)) and the other enriching for methylated DNA (HpaII cleaves unmethylated sequences). As McrBC cleaves between two methylated cytosine residues, both of which must be (G/A)^{me}C, it is useful in enriching the unmethylated fraction of DNA by subtraction, and has been used to evaluate methylation patterns genome-wide (Irizarry et al., 2008; Lippman et al., 2005). However, because there is no methylation-insensitive reference, detected differences in methylation may just as easily be caused by sequence polymorphisms that cause copy number variations or affect enzyme recognition sites in one or both samples.

An alternative approach developed in our laboratory, the HELP assay (HpaII tiny fragment enrichment by ligation-mediated PCR) (Khulan et al., 2006), relies on methylation-sensitive restriction enzyme digestion. Like other methylation-sensitive restriction enzyme microarray-based approaches, HELP uses comparative hybridization of digests from the restriction enzyme isoschizomers, HpaII and MspI. The HpaII signal channel reveals patterns of methylation and enriches specifically for hypomethylated sequences, while the MspI signal channel determines all potential HpaII/MspI cut sites and therefore serves as an internal control for sequence polymorphisms, copy number variations, and amplification efficiency. Like all the other restriction-based assays, HELP is only able to assess methylation status for a fraction of sites throughout the genome, corresponding to the HpaII recognition sequence (5'-CCGG-3').

Finally, there are a handful of additional approaches that are able to determine global levels of DNA 5-methylcytosine content. Enzymatic hydrolysis followed by liquid chromatography and mass spectrometry (LC-MS) enables quantitative determination of 5-methylcytosine levels in genomic DNA (Friso et al., 2002) and we have used this approach to establish the absence of DNA methylation in apicomplexan parasites such as *Toxoplasma gondii* (Gissot et al., 2008). The luminometric methylation assay (LUMA) is able to determine global methylation levels by pyrosequencing of overhangs generated by methylation-sensitive restriction enzymes (e.g. HpaII) compared to methylation-insensitive references (e.g. EcoRI or MunI, which digest 5'-GAATTC-3' and 5'-CAATTG-3', respectively) (Karimi et al., 2006a; Karimi et al., 2006b). LUMA has the added benefit of an internal control (i.e. the methylation-insensitive restriction enzyme digestion) and is therefore able to determine relative levels of methylation between samples, independent of varying DNA quantities.

b. Bisulphite Conversion Techniques—The gold standard technique to assess DNA methylation status is bisulphite sequencing, which relies on the conversion of cytosine residues to uracil in single stranded DNA following treatment with sodium bisulphite. The protocol consists of a few important steps: 1) covalent attachment of bisulphite to the 5-C of cytosine; 2) hydrolytic deamination of the resulting cytosine-bisulphite derivative to make uracil-bisulphite; and 3) removal of the sulphonate group by alkali treatment, generating uracil (Clark et al., 1994). Although 5-methylcytosine can also react with bisulphite reagent, the reaction is extremely slow and the equilibrium constant highly favors unconverted 5-methylcytosine rather than the deaminated product, thymine (Hayatsu, 1976). Because the bisulphite reaction generates non-complementary sequences (due to C:G conversion to U:G mismatches), a DNA region of interest must be amplified by PCR using locus and strand-specific primer sets. Thereafter, bisulphite-converted products can be analyzed using conventional sequencing (either direct (Dikow et al., 2007) or clonal (Frommer et al., 1992)), pyrosequencing (Dupont et al., 2004), or MassArray (Ehrich et al., 2005). Pyrosequencing and MassArray each reveal quantitative ratios of methylated and unmethylated DNA within a population of molecules, whereas direct clonal sequencing reveals methylation patterns on a single copy of DNA and therefore must be performed in many replicates to achieve the same degree of quantitative resolution. However, unlike direct sequencing, neither pyrosequencing nor MassArray are able to determine methylation haplotypes (i.e. patterns of methylation on a single molecule of DNA). Each of these techniques has proven to be reliable and accurate for single-locus studies, but each assay is limited in genomic coverage from ~20-50 bp (pyrosequencing) to ~500-600 bp (MassArray and direct sequencing).

A number of additional assays also make use of bisulphite conversion as a means of assessing DNA methylation status; however, these techniques do not possess the degree of quantitative resolution present in the aforementioned approaches. Methylation-specific PCR is a standard PCR reaction performed with two primer pairs, one of which is specific for bisulphite-converted methylated DNA and the other of which selectively amplifies bisulphite-converted unmethylated DNA (Herman et al., 1996). Relative comparison of the two amplifications can give an idea of the extent of methylation at a given locus, although a number of potentially confounding factors must be considered with each experiment (Ammerpohl et al., 2009). Methylation-specific PCR can also be applied to tissue sections *in situ* (Zhang et al., 2004). A related approach, MethyLight relies on amplification of bisulphite-converted DNA, however it is modified to make use of TaqMan probes to quantitatively determine specific methylation states within the amplicon sequence (Eads et al., 2000). On the other hand, CoBRA (Xiong and Laird, 1997) and Bio-CoBRA (Brena et al., 2006) measure methylation status using restriction enzymes that can recognize bisulphite-converted DNA sequence, where the recognition site is designed to contain the CG(s) of interest in the absence of other cytosines (e.g. BstUI cutting CGCG). Similar to CoBRA, methylation sensitive single nucleotide primer extension (Ms-SNuPE) analysis is limited to single CG dinucleotides, although it is not

confined to measuring methylation at restriction enzyme sites because it employs radioactive nucleotide incorporation as an alternative readout (Gonzalzo and Jones, 2002).

High-throughput bisulphite-based approaches such as BeadChip are designed to multiplex methylation-sensitive PCR reactions on a massive scale (Bibikova et al., 2006). Combined with GoldenGate microarray technology (Illumina), this assay is able to interrogate simultaneously individual CG dinucleotide methylation status at almost 30,000 predefined sites (Bibikova and Fan, 2009). An additional approach involves direct bisulphite sequencing of genomic DNA using massively-parallel sequencing technology. Direct sequencing of short stretches of bisulphite-converted DNA distributed throughout the entire genome has enabled high-resolution mapping of methylation patterns in plant genomes (Zhang et al., 2006; Zilberman et al., 2007) and in both pluripotent and differentiated mammalian cell types (Meissner et al., 2008). A number of practical problems currently hamper the accessibility and utility of these assays (Jeddeloh et al., 2008). As sequence read lengths are currently limited depending on the platform used, one limitation of this approach remains the ability to map bisulphite-converted (and therefore lower-complexity) sequence data back to unique sites within the genome. Other problems include significant cost as well as the creation of non-complementary forward and reverse strands which thereby double the effective size of the reference genome. However, increasing sequencer capacity is likely to improve the performance of this technology dramatically in future experiments.

c. Methods Using Direct Affinity—While analysis of bisulphite converted DNA is an indirect means of assessing DNA methylation that relies upon a chemical reaction, a number of proteins can be used experimentally to directly recognize methylated DNA. As discussed previously, many native proteins including the methyl binding domain proteins are able to recognize methylated cytosines. However, antibodies are also a part of the experimental toolbox, and can specifically recognize 5-methylcytosine residues with high affinity. Just as with tools relying upon bisulphite converted DNA, methylation affinity approaches have been applied to single locus as well as genome-wide studies. Practically, however, these approaches are often limited in resolution to DNA fragments that contain multiple methylated residues, often spanning DNA fragments between 200 and 1,500 bp in length. In order to measure protein occupancy of methylated DNA, these methylation affinity approaches must selectively capture methylated DNA fragments and separate them from unmethylated fragments. This is most often accomplished by precipitation techniques including purification with agarose or magnetic beads and other matrices. Depending on starting material, selective enrichment of methylated DNA can be followed by PCR amplification, most often used for preparation of samples for whole genome approaches.

Each approach relies on a different 5-methyl cytosine binding protein. Methylated DNA immunoprecipitation (MeDIP) employs a specific antibody that recognizes 5-methylcytosine (Jacinto et al., 2008), while a chimeric MBD2/IgG1 protein is used in methyl-CpG immunoprecipitation (Schilling and Rehli, 2007). Similarly, MIRA relies on a MBD2-glutathione-S-transferase chimera, which can bind sonicated methylated DNA through MBD2 and can be isolated along with its methylated DNA cargo by a glutathione sepharose matrix (Rauch and Pfeifer, 2005). In addition to MBD2 and specific antibodies, the methyl binding domain of MeCP2 has also been used for isolating methylated DNA for subsequent analysis (Yan et al., 2002). However, MBD proteins can introduce sequence-specific bias in methylation status due to their inherently variable affinity for methylated cytosines in different sequence contexts (Nan et al., 1997). Immunoprecipitation approaches (i.e. MeDIP) on the other hand are highly sensitive to and therefore biased by CG density. This becomes particularly problematic when these affinity-based approaches are used to generate methylated DNA fractions for whole-genome analysis (Irizarry et al., 2008). Whether hybridized to microarrays (Weber et al., 2005) or applied to massively parallel sequencing (Jacinto et al., 2008; Pomraning

et al., 2009), the low specificity and sensitivity of affinity-based techniques generally limit their value as a genome-wide assessment of cytosine methylation. However, when the studies are limited to CG-dense regions and test intergenomic differences in methylation, they should be reasonably powerful, while the development of new analytical approaches to account for CG dinucleotide heterogeneity (Down et al., 2008) may allow improved performance of this assay in the CG depleted majority of the genome.

2. Testing Histone Modifications and Chromatin Structure

A number of other tools are also available to study the epigenome at the histone and chromatin structural levels. Just as affinity-based approaches can be used to detect methylated DNA, antibodies are able to recognize a wide variety of specific histone modifications and DNA-binding proteins. Referred to as chromatin immunoprecipitation (ChIP), DNA can be enriched based on the modified histones or other proteins with which it is associated (Orlando and Paro, 1993). Subsequent steps are able to determine the positional identity of the associated DNA on both a locus-specific and genome-wide level. In combination with DNA microarrays (ChIP-chip) (Bernstein et al., 2002; Robyr et al., 2002), tag sequencing techniques (e.g. SACO, GMAT, and ChIP-PET) (Impey et al., 2004; Roh et al., 2004; Wei et al., 2006), or massively parallel sequencing (ChIP-seq) (Barski et al., 2007; Johnson et al., 2007; Robertson et al., 2007), chromatin immunoprecipitation techniques are able to measure a variety of epigenomic details, from specific histone modifications, histone variants, and other chromatin constituents. These approaches are powerful, but they rely on the specificity of the antibody or antibodies, with additional variability or bias introduced by prerequisite chromatin fragmentation techniques (e.g. sonication or micrococcal nuclease (MNase) digestion).

Chromatin accessibility can be determined by various reagents, including DNase, MNase, restriction enzymes, and a number of chemicals. Restriction enzyme cleavage may determine site-specific accessibility, where a failure to digest can be interpreted as a region of inaccessible chromatin. MNase digestion is more often used to define nucleosome boundaries. DNaseI on the other hand defines general accessibility of chromatin structure, and has been used in conjunction with high-throughput techniques to map regions of relatively open and closed chromatin (Crawford et al., 2006a; Crawford et al., 2006b). Another approach to profile nucleosome occupancy genome-wide is FAIRE, formaldehyde-assisted isolation of regulatory elements. In this ingenious assay, chromatin is cross-linked *in vivo*, sonicated to create fragments, and then extracted by phenol-chloroform to remove protein-bound material. The purified DNA in the aqueous phase is protein-free and is therefore enriched for nucleosome-free material which can be analyzed as single loci or using genome-wide approaches (Giresi et al., 2007).

Techniques to measure DNA interactions (e.g. enhancer elements) over distances spanning over 100 kb or even between two different chromosomes have shown promise in identifying a number of important gene regulatory phenomena (Lancot et al., 2007). Both fluorescence *in situ* hybridization (FISH) and chromosome conformation capture (3C) have conclusively demonstrated colocalization of distinct regions of DNA separated by long-distances (Dekker et al., 2002). Moreover, high-throughput extensions of 3C technology (e.g. 4C) are able to determine interaction profiles of a single locus with the entire remainder of the genome (Simonis et al., 2006; Zhao et al., 2006). The 5C technique is an additional extension of 3C, where multiplexed ligation-mediated PCR of standard 3C libraries can determine many interacting chromosome regions in parallel (Dostie et al., 2006).

Technological Considerations and Experimental Decisions

While the range of epigenetic regulatory mechanisms and analytical techniques available for their study is immense, there are practical constraints of sample preparation and amount that

constrain the choice available in practice. ChIP-based techniques currently require large numbers of cells, freshly-prepared and as dissociated from each other as possible, to allow homogenous cross-linking to occur. It is also difficult to decide which of the >60 post-translational modifications of histones or DNA-binding proteins to study in the absence of prior information (see Figure 2 for examples). At this exploratory stage of research into the role of epigenomic dysregulation in human disease, cytosine methylation remains the most commonly-used approach, as the covalent modification of the DNA molecule is extremely stable, and as few as single cells at a time can be studied (Kerjean et al., 2001).

Genome-wide approaches, whether one of the cytosine methylation assays or ChIP-based, are generally only semi-quantitative at best. Shotgun bisulphite genomic sequencing is genome-wide and quantitative but is limited by being too expensive at present for the analysis of multiple samples. When designing a study to test for epigenomic dysregulation in a human disease or ageing, a genome-wide, semi-quantitative approach is worth performing on a subset of samples, defining those loci at which the dysregulation is more consistently occurring. The second stage of the experiment is to perform single-locus, quantitative studies on these and a further panel of samples to validate the findings at these loci. Such a two-stage design is at present the most practical means of performing studies that are comprehensive, genome-wide and still quantitative.

An issue that arises is the power of the semi-quantitative genome-wide assay to detect the epigenetic differences occurring in the test compared with the control cells. While most assays should be easily able to distinguish between epigenetic regulatory marks present in 100% of the alleles in a cell population and 0% in another population, it appears that such extreme differences may not be the rule but may instead be the exception. Some recent studies have shown differences in cytosine methylation between cohorts that are in the range of 10%, the example being a study of adults who had been exposed to famine while *in utero* (Heijmans et al., 2008). It is difficult to understand how such differences exert functional effects of consequence, but even if this example is not typical, we need to consider the discriminatory power of the genome-wide assay in combination with our expectation of likely degrees of difference in the epigenetic regulatory pattern if we are going to be able to perform power calculations for these studies.

A problem with ChIP-chip or ChIP-seq is that these genome-wide assays currently generate a binary output, the presence or absence of the chromatin constituent or modification at a site, and not a direct indication of whether this is present in more or fewer alleles in one cell sample compared with another. As such, these assays are best used for the identification of extreme differences and make power calculation difficult. Cytosine methylation assays have not generally been described in terms of their quantitative discriminatory capacities, with the exception of a recent massively-parallel sequencing-based approach described by Ball *et al.* (Ball et al., 2009). Their methyl-sensitive cut counting (MSCC) assay appears to be capable of discriminating levels of methylation of as little as 20-30%, and certainly differences of $\geq 40\%$. Based on this quantitative discriminatory capacity, we calculate that the use of the MSCC assay in a situation requiring the discrimination of 20% methylation differences would require 100 samples per group to achieve adequate statistical power following compensation for the multiple testing involved (MJF, manuscript in preparation). While clearly this is an extreme example, it serves as a guide for the likely required magnitude of these epigenome-wide association studies, comparing favorably with the greater sample requirements for genome-wide association studies.

Epigenetic Dysregulation and Aging

The relationship between epigenetics and aging has been studied for over 40 years, when it was first determined that spawning fish showed global decreases in DNA methylation with increasing age (Berdyshev et al., 1967), subsequently reproduced in other animals as well as in human peripheral blood cells (Fuke et al., 2004). Attention began to be paid to possible chromatin-mediated mechanisms over a decade ago (Howard, 1996), following which some early chromatin immunoprecipitation-based assays revealed evidence for regional changes of histone H4 acetylation as part of cellular aging (Russanova et al., 2004a; Russanova et al., 2004b). Global hypomethylation was also observed in both replicative and premature peroxide-induced senescence in human embryonic lung fibroblasts (Zhang et al., 2008), whereas immortal cells were resistant to changes in DNA methylation (Wilson and Jones, 1983). Similar results were demonstrated for human diploid fibroblast (MRC-5) cells, moreover experimentally-induced hypomethylation shortened their *in vitro* lifespan by 25% (Fairweather et al., 1987). While cell culture-based studies provided experimentally controlled environments, later clinical studies revealed similar phenomena. A longitudinal analysis of over 1,000 individuals (from 55 to 92 years of age) identified significant and progressive decreases in methylation at Alu repetitive elements distributed throughout the genome (Bollati et al., 2009). Parkinson's disease patients, a distinctive model of age-associated degeneration, also show DNA hypomethylation, particularly in subtelomeric chromosomal regions (Maeda et al., 2009).

However, studies have also identified hypermethylation with increasing age in ribosomal DNA from liver and sperm cells of senescent rats (Oakes et al., 2003) as well as a number of CpG island-containing promoters in humans (Calvanese et al.; So et al., 2006). Locus-specific hypermethylation of a transcription factor, *TCF4*, caused decreases in its expression associated with increasing age of normal gastric mucosa (Kim et al., 2008). Additionally, DNA methylation analysis of skeletal muscle biopsies from non-diabetic elderly and young individuals revealed promoter hypermethylation and correspondingly decreased expression of the *COX7A1* gene in accordance with a downregulation of oxidative phosphorylation in elderly muscle (Ronn et al., 2008). Furthermore, in senescent cell lines (i.e. IMR-90), DNA hypermethylation was associated with specific loci, in particular protein kinase CK II? (Kim et al., 2009). In aging rats, DNA hypermethylation was observed at the glucokinase (*GCK*) promoter in association with decreased hepatic Gck expression and progressive age-associated decline in hepatic glycogen content (Jiang et al., 2008). Rat kidneys also demonstrate age-dependent alterations in DNA methylation, with N-cadherin promoter hypermethylation in old rats (Akintola et al., 2008). However, no corresponding changes were observed for N-cadherin in rat liver, implicating that these age-dependent epigenetic changes are tissue-specific.

Studies in monozygotic (MZ) twins reveal the extent to which epigenetic changes may occur with increasing age. Although MZ twins typically share near-identical copies of their DNA, it appears that aging is a very heterogeneous, individual-specific process and that genetics likely accounts for only a fraction of the phenotypic variability observed (e.g. different onset and extent of cataracts, gray hair, and osteopenia) (Gurland et al., 2004; Karasik et al., 2004). The extensive differences in DNA methylation in MZ twins are likely to play an important role in establishing these phenotypic distinctions (Kaminsky et al., 2009). Indeed, a genome-wide epigenetic study of young and old MZ twins revealed that elderly twins exhibited highly variable epigenotypes, particularly at repetitive DNA elements or other non-promoter sequences (Fraga et al., 2005). Moreover, this increasing epigenetic variability occurred as a function of increasing age. Thus it appears that epigenetic changes accumulate over time, and can account for observed inter-individual differences in expression and phenotype (Cheung et al., 2003; Fraga et al., 2005; Sharma et al., 2005). A 10-year longitudinal study in two populations ($n_1=111$, $n_2=126$) confirmed that intraindividual DNA methylation changes over

time and that a genetic factor may influence an individual's ability to maintain methylation states over time (Bjornsson et al., 2008). While differing environments may play a role, the phenotypic contributions of epigenetic discordance in MZ twins is supported by observations in isogenic laboratory strains. These genetically identical animals, housed under the same stringently controlled environment, exhibit significant epigenetic differences associated with distinct phenotypes (Gartner, 1990; Vogt et al., 2008).

It is not difficult to imagine the epigenetic regulation of a locus becoming altered over time, following a series of cell divisions. Experiments to clone single cells and to expand them in culture have been performed, allowing measurement of cytosine methylation, a major epigenetic regulator, in the populations of cells obtained and the deviation from the possible 0, 50 or 100% methylation at a given locus. These are the only three possibilities, as the cytosine on each chromosome can be either methylated or not. In separate studies the error rate was calculated to be somewhere between <1% (Ushijima et al., 2003) and 4% (Laird et al., 2004) per cell division at a given position. The data also indicated that the error rates differed depending on genomic context, with cytosines located outside CpG islands having twice the error rate of those in the more cytosine-guanine (CG) dinucleotide-rich CpG island sequences (Ushijima et al., 2003). With error rates of this magnitude, it is perhaps surprising that cells manage to maintain tissue-specific patterns of methylation, and is consistent with the intermediate methylation values that are frequently noted (Ordway et al., 2007).

The model of random drift from a starting epigenetic state is interesting in terms of environmental responses and ageing. If a population of cells is inherently heterogeneous in terms of its epigenetic organization, this may allow a range of states to exist that will include some more capable of responding to new environmental influences. Over time, if this drift starts to include stem cells, this could help to explain fixed age-related changes in the epigenome. In such a model the presence of a DNA mutation that causes one cell clone to outgrow others within the population could be viewed as a driver of epigenetic state change for the population, and may be consistent with the association of cell cycle control genes with age-related diseases such as type 2 diabetes mellitus (Zeggini et al., 2007). The field of ageing and the epigenome is likely to be especially rewarding and revealing, both on a whole organism level as well as cellular selection and cell population dynamics.

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Figure 1.

Cytosine methylation can be studied a number of ways, but the assays mostly fall into the three categories shown. Restriction enzymes can selectively digest unmethylated or methylated DNA, affinity reagents can also bind selectively to each methylation state, while chemical mutagenesis with sodium bisulphite preferentially deaminates unmethylated cytosine to generate uracil, with methylcytosine being relatively protected. In all cases it becomes necessary to map the products of the initial enrichment, selection or chemical conversion to determine where in the genome the methylated cytosines are located, usually involving microarrays or massively-parallel sequencing.

H1

S E T A P A A P A A P A P A E K T **P** V K K K A R K S A G A A K R K A S
 G P P V S E L I T K A V A A S K E R S G V S L A A L K K A L A A A G Y D
 V E K N N S R I K L G L K S L V S K G T L V Q T K G T G A S G S F K L N
 K K A A S G E A K P K A K K A G A A K A K K P A G A A K K P K K A T G A
 A T **P** P K K S A K K T P K K A K K P A A A A G A K K A K S P K K A K A A
 K P K K A P K S P A K A K A V K P K A A K P K T A K P K A A K P K K A A
 A K K K

H3

A R **Me** T **P** K **Me** Q T A R K **AcMe** S **P** T **P** G G K **AcMe** A P R **Me** K **Ac** Q L A T K **AcMe** A
 A R **Me** K **AcMe** S **P** A P A T **P** G G V K **Me** K **Me** P H R Y R P G T V A L R E I R R Y
 Q K S T E L L I R K L P F Q R L V R E I A Q D F K **Me** T D L R F Q S S A V
 M A L Q E A C E A T L V G L F E D T N L C A I H A K **Ac** R V T **P** I M P K **Ac**
 D I Q L A R **Me** R I R G E R A

H4

S **P** G R **Me** G K **Ac** G G K **Ac** G L G K **AcMe** G G A K **Ac** R H R K **AcMe** V L R D N I Q
 G I T K P A I R R L A R R G G V K R I S **P** G L I Y E E T R G V L K **Me** V F
 L E N V I R D A V T Y T E H A K **Ac** R K **AcMe** T V T A M D V V Y A L K R **Me** Q
 G R T L Y G F G G

H2A

S **P** G R G K **Ac** Q G G K **Ac** A R A K **Ac** A K **Ac** T R S S R A G L Q F P V G R V H
 R L L R K **Ac** G N Y S E R V G A G A P V Y L A A V L E Y L T A E I L E L A
 G N A A R D N K K T R I I P R H L Q L A I R N D E E L N K **Me** L L G K **Me** V
 T I A Q G G V L P N I Q A V L L P K K **AcU** T E S H H K A K G K

H2B

P E P A K **AcMe** S A P A P K K **Ac** G S **P** K **Ac** K A V T K **Ac** A Q K **Me** K **Ac** D G K K
 R K R S **P** R K E S **P** Y S V Y V Y K **Me** V L K Q V H P D T G I S S K A M G I M
 N S F V N D I F E R I A G E A S R L A H Y N K **Ac** R S T I T S R E I Q T A
 V R **Me** L L L P G E L A K **Ac** H A V S E G T K **Ac** A V T K **AcU** Y T S S K

Ac Acetylation
Me Methylation
P Phosphorylation
U Ubiquitination

Figure 2.

Four post-translational modifications of specific amino acids of the linker (H1) or core histones are illustrated to demonstrate the potential combinatorial complexity that they confer.