REVIEW

Epigenetics, Plasticity, and Evolution: How do We Link Epigenetic Change to Phenotype?

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ABSTRACT

Epigenetic mechanisms are proposed as an important way in which the genome responds to the environment. Epigenetic marks, including DNA methylation and Histone modifications, can be triggered by environmental effects, and lead to permanent changes in gene expression, affecting the phenotype of an organism. Epigenetic mechanisms have been proposed as key in plasticity, allowing environmental exposure to shape future gene expression. While we are beginning to understand how these mechanisms have roles in human biology and disease, we have little understanding of their roles and impacts on ecology and evolution. In this review, we discuss different types of epigenetic marks, their roles in gene expression and plasticity, methods for assaying epigenetic changes, and point out the future advances we require to understand fully the impact of this field. *J. Exp. Zool. (Mol. Dev. Evol.) 322B:208–220, 2014.* © 2014 The Authors. *J. Exp. Zool. (Mol. Dev. Evol.)* published by Wiley Periodicals, Inc.

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The term "epigenetics" has a complex history. Originally meant to refer to the mechanisms that link gene to phenotype (Waddington, '42), it has, in recent years, become more narrowly defined to refer only to modifications of the DNA and chromatin that do not change the underlying DNA sequence. This has led to a focus on DNA modifications, such as the reversible addition of a methyl group to a cytosine residue to generate 5-methylcytosine, and post-translational modification of histone proteins (Fig. 1). These epigenetic mechanisms, which are linked to more familiar aspects of gene regulation by proteins such as transcription factors, act to regulate gene expression in cells. Through regulation of gene expression, epigenetic mechanisms have the potential to define and alter cell phenotypes and, as the epigenome can be altered by the environment (i.e., Dolinoy et al., 2007; Sinclair et al., 2007; Kucharski et al., 2008; Seong et al., 2011; Gertz et al., 2012; Herb et al., 2012; Wang et al., 2012), may also orchestrate dynamic regulation of the genome in response to changes in the environment. Epigenetic mechanisms also mediate dosage compensation, chromosomal silencing and imprinting (Trescot et al., 2006; Wutz and Gribnau, 2007; Abramowitz and Bartolomei, 2012; Gertz et al., 2013).

Epigenetic mechanisms are intimately linked with cell differentiation (reviewed in Reik, 2007). In vitro experiments have demonstrated that as cells move from a pluripotent to a terminally differentiated state, epigenetic marks change across the genome (Hochedlinger and Plath, 2009). In vertebrates these epigenetic marks are found across all regions of the genomic landscape including enhancer, promoter and intergenic regions of the genome, as well as in exons and introns. How and when these

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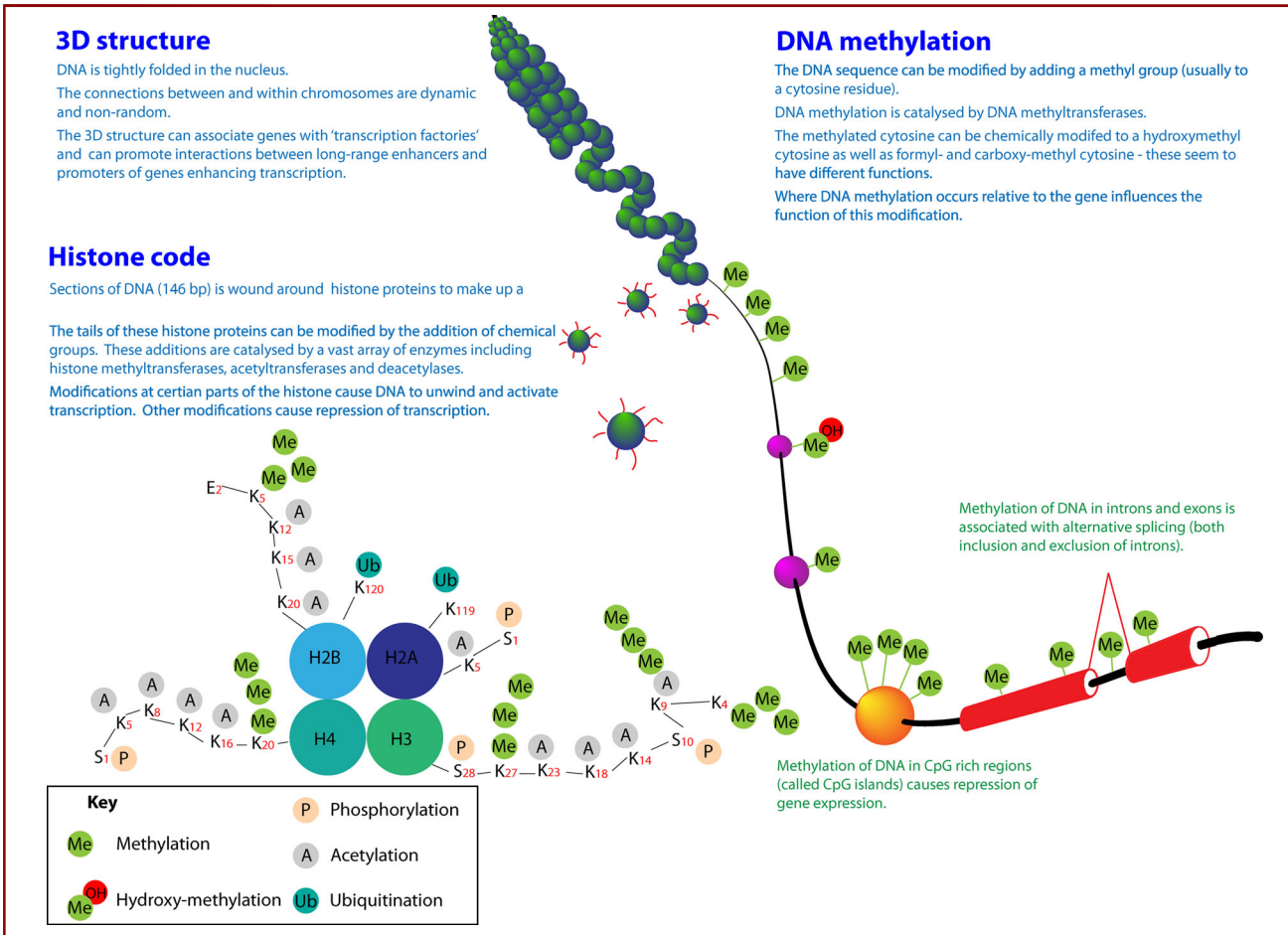

Figure 1. Types of epigenetic modifications and their potential effects on gene expression and chromatin structure.

epigenetic landscapes are being established is just beginning to be understood (i.e., Ziller et al., 2013). Within a multicellular organism it is critical to distinguish between those epigenetic changes that reflect cell-type specific changes related to cell differentiation or are constitutive (e.g., related to sex determination), from those epigenetic changes induced by environmental exposures.

One potentially important role of epigenetic mechanisms appears to be a cell's way of remembering a past gene-regulatory event, or holding one in reserve until it is needed. Because of this ability to stably "remember" a gene regulatory event across the lifespan of an organism, and, possibly, across generations (Anway et al., 2005; Stouder and Paoloni-Giacobino, 2010; Greer et al., 2011; Stouder and Paoloni-Giacobino, 2011; Ashe et al., 2012; Manikkam et al., 2012a), epigenetics has become vital to our understanding of biology, ecology, and evolution.

TYPES OF EPIGENETIC MODIFICATIONS

DNA methylation involves the modification of a DNA base, most often a cytosine in a CpG dinucleotide pair, with the addition of a methyl group thus affecting the coiling of DNA around histones and changing the potential binding of transcriptional factors in part by recruiting methyl CpG binding proteins (MCBPs). Although absolute levels of DNA methylation vary between species and cell types (Lister et al., 2009; Feng et al., 2010a; Zemach et al., 2010; Nanty et al., 2011), in humans there is experimental evidence for 80–96% of the CpG residues in the genome being methylated under various conditions (Varley et al., 2013; Ziller et al., 2013). Much of our understanding of the function of DNA methylation has come from imprinting in mammals (reviewed in Abramowitz and Bartolomei, 2012) and the study of cancer cell lines (reviewed in Laird and Jaenisch, '96), where DNA methylation is often aberrant, both in placement, and in pattern (Miremadi et al., 2007; Cedar and Bergman, 2012).

Previous studies have focused on the role of DNA methylation in generally repressing gene expression through methylation of CpG islands near promoters of genes (Jones, 2012). DNA methylation is found throughout genes, not just in promoter regions, in animals and plants (Feng et al., 2010a; Zemach

Table 1. Summary of epigenetic marks, techniques that can be used to interrogate them and key questions that need to be addressed concerning the function and regulation of these marks.

	Function	Techniques loci specific	Techniques whole genome	Key questions
DNA methylation				
Dnmt1a Dnmt1b	Maintenance of methylation marks across cell divisions	Bisulfite conversion followed by Sanger sequencing HRM MethylLight epiTYPER	Bisulfite conversion followed by Reduced representation bisulfite sequencing Shot-gun sequencing MeDIP	How is the specificity of DNA methylation marks conferred? What regulates the turnover of methylation marks?
Dnmt3	Establishment of DNA methylation marks de novo	Bisulfite independent methods Methylation sensitive restriction digestion (coupled with PCR and/or sequencing) Immunoprecipitation (MeDIP) (coupled with PCR and/or sequencing)		Which, if any, methylation marks are transmitted via the germline?
Tet1	Conversion of 5′ methylcytosine to 5′ hydroxymethylcytosine	Methylation sensitive restriction digestion (coupled with PCR and/or sequencing) Immunoprecipitation (MeDIP) (coupled with PCR and/or sequencing) TAB-seq (TET-assisted bisulfite sequencing)		What controls the specificity of DNA demethylation?
Chromatin structure/function				
Histone modifications		Chromatin immunoprecipitation (with antibody to the modification of interest) coupled to qPCR.	Chromatin immunoprecipitation (with antibody to the modification of interest) with Next Generation Sequencing (ChIP-seq) or hybridization to a microarray (ChIP-chip).	How do histone modifications interact with other epigenetic systems?
3-dimensional structure		Chromatin conformation capture (3C) which uses qPCR to detect the interactions between 2 known loci and derivatives, including 4C (1 known loci vs. whole genome).	5C, 6C, Hi-C, and ChIA-PET.	What determines the 3D structure of the genome? Is genome structure dynamic? Is it sensitive to environmental perturbation?

et al., 2010; Sarda et al., 2012). Promoter methylation appears to have evolved in the vertebrate lineage whereas methylation of gene bodies was likely present in the last common ancestor of plants and animals (Feng et al., 2010a; Zemach et al., 2010). It seems that the position of DNA methylation relative to the gene (i.e., intron, exon, transcriptional start site, or promoter) determines how gene transcription is affected by methylation (Jones, 2012). For instance, gene body methylation has multiple functions including repressing intragenic promoter activity (Maunakea et al., 2010), alternative splicing (Lyko et al., 2010; Shukla et al., 2011; Foret et al., 2012; Sati et al., 2012) and controlling transcriptional elongation (Lorincz et al., 2004) ensuring that the first and last exons are included in a transcript (Sati et al., 2012), while DNA methylation at the 5′ end of the gene is associated with transcriptional silencing (Brenet et al., 2011).

DNA methylation is established and maintained by two families of DNA methyltransferase enzymes: DNMT1 and DNMT3 (reviewed in Goll and Bestor, 2005). We do not understand how, or indeed if, the DNA methyltransferase enzymes are targeted to particular sites in the genome to provide specificity for DNA methylation, although it appears that non-coding RNAs may play a central role. In plants, DNA methylation can be targeted to specific genomic loci by RNA molecules (RNA directed DNA methylation, RdDM) (Mahfouz, 2010; Zhang and Zhu, 2011). Some evidence supports the role for RNA, specifically small RNAs, in directing methylation in animals (Weinberg et al., 2006; Aravin and Bourc'his, 2008; Holz-Schietinger and Reich, 2012).

Demethylation, the removal of a methyl group from a cytosine residue, had been assumed to be a passive process, via loss of methylation marks across cell divisions, but it has now been shown to occur independent of cell division (i.e., Mayer et al., 2000; Oswald et al., 2000). Demethylation of DNA can occur via DNA repair pathways mediated by Gadd45 (growth arrest and DNA damage inducible protein 45) (Barreto et al., 2007; Ma et al., 2009a; Niehrs, 2009; Niehrs and Schafer, 2012). Gadd45 can be induced by external stimuli (i.e., Ma et al., 2009b) and appears to target specific genes for demethylation (Jin et al., 2008; Engel et al., 2009; Schafer et al., 2010).

A second pathway for demethylation of DNA employs TET (ten eleven translocation) enzymes, which convert 5-methylcytosine to 5-hydroxymethyl cytosine (Tahiliani et al., 2009), which is then processed to 5-formylcytosine and 5-carboxylcytosine (He et al., 2011; Ito et al., 2011). Both these derivatives act as substrates for a thymine-DNA glycosylase, which results in the regeneration of a non-methylated cytosine (He et al., 2011; Maiti and Drohat, 2011). The biological functions of the derivatives of 5-methylcytosine are unknown, but in human cells each associates with proteins not linked to DNA repair, implying that these derivatives may also act as epigenetic marks that recruit transcriptional regulators (Spruijt et al., 2013). As with methylation enzymes, we do not understand how demethylation enzymes are targeted to specific regions of the genome.

Nuclear chromatin is organized into nucleosomes; a segment of DNA wound around eight core histone proteins. These proteins are extensively post-translationally modified (reviewed in Peterson and Laniel, 2004) by a suite of enzymes (Biel et al., 2005; Marmorstein and Trievel, 2009) that are temporally and developmentally regulated (Lin and Dent, 2006; Heintzman et al., 2009; Kharchenko et al., 2011; Dunham et al., 2012; Pengelly et al., 2013). The post-translational modification of these histone proteins is known to regulate gene-expression by altering the accessibility of the underlying DNA to transcription factors (Wu et al., '79; Bell et al., 2010). It has been proposed that histone modifications may act as a signal integration and storage platform, allowing cells to record and store signaling events, including environmental signals (Badeaux and Shi, 2013).

We are beginning to understand how histones at specific loci may be targeted by histone modifying enzymes. Specific DNA sequences have been identified that recruit histone modifying enzymes (Fritsch et al., '99; Tillib et al., '99; Klymenko et al., 2006) and long non-coding RNAs have also been proposed to have a role in targeting modification of histones associated with particular loci (Tsai et al., 2010; Spitale et al., 2011). There is increasing evidence of cross-talk between DNA methylation and histone modifications (i.e., Hashimshony et al., 2003; Bartke et al., 2010; Hagarman et al., 2013; Spruijt et al., 2013) supporting the idea that these mechanisms act together to regulate gene expression. It is not known how cross-talk between these two systems is mediated, but data implies that, in at least some circumstances, changes to histone modifications may be induced prior to methylation changes that then serve as more stable epigenetic marks (Park et al., 2008).

In addition to these "classical" epigenetic systems, small RNA molecules, such as small interfering RNA (siRNA) and piwi-RNA (piRNA), have epigenetic potential. siRNAs are 21–22 nucleotides in length and are produced from endogenous double stranded RNA. These molecules associate with Argonaute proteins that induce localized chromatin remodelling (Fagegaltier et al., 2009; Burkhart et al., 2011) and may maintain genes in a "poised" state, ready to be activated (Cernilogar et al., 2011). piRNAs are larger (23–29 nucleotides) and are produced by a different mechanism to siRNAs (reviewed in Castel and Martienssen, 2013). piRNAs were initially discovered in germ-line cells, but are now known to be widely distributed throughout somatic tissues (Yan et al., 2011; Ishizu et al., 2012). In the germ-line, piRNAs mediate transposon silencing via chromatin remodeling (Brower-Toland et al., 2007; Wang and Elgin, 2011).

Epigenetic marks may also mediate the way that DNA is organized in three-dimensional space within the nucleus of a cell. This structure can bring enhancer and promoter elements into contact, or can recruit genes to "transcription factories" facilitating gene expression. It means that both genetic polymorphisms and epigenetic polymorphisms in regulatory regions of the genome have the potential to act in both *cis* and *trans* to affect

gene transcription. Looping can also allow interactions with insulator elements causing repression of gene expression. Compartmentalization of the genome in three-dimensions is dynamic and is associated with cell type specific gene expression patterns (Lieberman-Aiden et al., 2009; Varley and Mitra, 2010). It is unknown whether the association of higher order chromatin structures with particular histone modifications are a cause or consequence of those higher order structures (Greer and Shi, 2012).

EPIGENETICS AND PHENOTYPIC PLASTICITY

Phenotypic plasticity, the ability of an individual genome to produce different phenotypes when exposed to environmental cues (Pigliucci et al., 2006), is widespread amongst both plants and animals.

Well-known examples of phenotypic plasticity include caste polyphenisms in social insects, seasonal polyphenisms in butterflies and as well as the mechanisms of learning and immune system adaptation (Fusco and Minelli, 2010). Epigenetic changes have been associated with polyphenisms like caste development in the honeybee (Kucharski et al., 2008) and ants (Bonasio et al., 2012; Simola et al., 2013) and phenotypic plasticity in mammals; specifically maternal mood in humans, affecting methylation of the glucocorticoid receptor (Oberlander et al., 2008), and differential methylation of genes in the umbilical cord being associated with *in utero* growth (Lim et al., 2012) and childhood adiposity (Godfrey et al., 2011).

Predictive adaptive responses (PARs) are a subclass of phenotypic plasticity where animals in early life make predictions about their future environment based on environmental cues received early in development (Gluckman et al., 2005). Classical examples of PARs include the meadow vole, which receives environmental cues *in utero*, via maternal melatonin levels, about the season it is gestating toward and the infant is born with a thicker coat in autumn than it is in spring (Lee and Zucker, '88). PARs are believed to be established via epigenetic marks established by a triggering cue in early development that becomes a proxy for predicting a subsequent environment. In turn these epigenetic marks affect the physiological trajectory that is followed as the organism develops—establishing effects on metabolism or morphology that may have positive effects on fitness as the organism survives to reproduce (Varley et al., 2009). Yet these marks are carried beyond peak reproduction through that organism's life and this, together with the probabilistic nature of early prediction of later-life environments, may have consequences that have been extensively discussed in relation to non-communicable disease in humans.

Predictions that an animal makes about its future environment may not always be correct, and the idea of mismatch underpins the "Developmental origins of Human Disease" paradigm (Gluckman and Hanson, 2006). This describes how the environment influences gene expression, possibly via epigenetic mechanisms, in the fetus and infant that are then stable throughout an individual's life. It is proposed these predictions evolved to enhance survival to reproduction, even if they can become disadvantageous in later life (Bateson et al., 2004; Gluckman and Hanson, 2004a, 2004b, 2005). Evidence is building in clinical studies that epigenetics underpins, or is at least associated with, this concept of fetal programming, hypothesized to be partly responsible for the current burden of non-communicable diseases, such as metabolic syndrome (Hanson and Gluckman, 2008; Gluckman et al., 2010; Hanson et al., 2011).

Evolutionary Implications

Two recent findings have radically expanded the possible role of epigenetics in evolution and ecology. Firstly, in some situations, environmental cues can influence epigenetic programming (i.e., Dolinoy et al., 2007; Sinclair et al., 2007; Kucharski et al., 2008; Seong et al., 2011; Gertz et al., 2012; Herb et al., 2012; Wang et al., 2012), and secondly, this information has the potential to be passed on to the subsequent generations via the gametes (Anway et al., 2005; reviewed in Jablonka and Raz, 2009; Stouder and Paoloni-Giacobino, 2010; Greer et al., 2011; Stouder and Paoloni-Giacobino, 2011; Ashe et al., 2012; Manikkam et al., 2012a). This expanded view raises the possibility that these marks may be able to produce genetic change over time periods that may be relevant to evolution. The idea that epigenetic marks may carry gene expression changes across generations, probably in a limited way, is of importance in our understanding of the genetic assimilation of acquired traits (Bateson and Gluckman, 2011).

We are coming to understand that the genome is an exquisitely regulated structure, with various regions and domains being accessible to transcription at any time and in any cell. This structure is maintained by many complex mechanisms, including epigenetic ones, but also the related three-dimensional organization of the genome within the nucleus. With this understanding the genome is perhaps well explained by Waddington's "epigenetic landscape." Perturbations of this landscape through anticipated environmental changes, as in the case of a polyphenism, a continuous reaction norm, or in a PAR, leads to modifications in the landscape, and new phenotypic outcomes becoming favoured. In the case of a more disrupting influence, such as a heat shock in the case of genetic assimilation, the epigenetic landscape may be modified to buffer against damage, and some of that modification may be passed on to future generations through the inheritance of those epigenetic marks.

Numerous authors (i.e., Bunt et al., '88; West-Eberhard, 2005a, b; Pigliucci et al., 2006), have suggested that plastic changes somehow prefigure genetic ones, that then stabilize an environmentally-induced trait in future generations. It seems likely that if such processes exist they involve molecular epigenetic mechanisms (Feinberg and Irizarry, 2010; Bateson and Gluckman, 2011). One feature of methylated cytosine residues is that they are susceptible to deamination, and have a higher mutation rate to thymine than non-methylated bases. Is it possible that this

hypermutability provides a mechanism by which epigenetic changes may lead to genetic ones? (Bateson and Gluckman, 2011). While this is an attractive idea, it is important to note that the epigenetic change and subsequent mutations must occur in the germ-line to have any importance to evolution. This is a critical point as, if all cell types have their own epigenetic landscapes, a change occurring in some cell type having a beneficial effect, would also have to occur in the germ line to have a trans-generational or evolutionary effect.

Mechanisms for Transmission of Epigenetic Information. Indirect epigenetic inheritance occurs when an environmental cue induces a behavior or physiology, via epigenetic marks, that then induces the same epigenetic mark and associated behavior in subsequent generations. The cue is passed behaviorally or physiologically between generations, not via *trans*-meiotic passage of the epigenetic mark. An example of this mechanism is the epigenetic change in neuro-hormonal pathways in the mouse by altered maternal grooming that lead the offspring to grow up with the same maternal behavior that again induces the same epigenetic mark and thus behavior in the next generation (Weaver et al., 2004).

Direct transmission of epigenetic information between generations can occur, where the environmental influence directly affects the germline of the parent, or is mediated via interactions between the somatic cells and germline (reviewed in Jablonka and Raz, 2009). In both cases in order for true transgenerational transmission of environmental information, epigenetic marks must be stable and heritable through meiosis (Osbourne et al., in press).

The DNA methylation landscape can be retained through mitosis via the activity of the DNMT1 proteins, or maintenance methyltransferases. During meiosis, and then embryonic development, there is substantial reprogramming of DNA methylation of both sperm and oocytes in vertebrate model species, although a number of loci are protected (Feng et al., 2010b; Seisenberger et al., 2012; Jiang et al., 2013; Potok et al., 2013). This provides the potential for environmentally induced DNA methylation patterns to be transmitted to the next generation during gametogenesis. Consistent with this, environmental exposure to particular chemicals is associated with altered patterns of DNA methylation, particularly in the sperm (i.e., Guerrero-Bosagna et al., 2010; Manikkam et al., 2012a,b; Tracey et al., 2013).

The histone modification landscape can also be retained, fully or partially, through cell division although we do not understand how this occurs. It may be that the "cross talk" between DNA methylation and histone modifications is integral for this process. Methylation marks, transferred via maintenance methyltransferases, may be used as a "template" to establish the histone modification landscape de novo in the new copy of the DNA. However, animals such as *Drosophila melanogaster* and *Caenorhabditis elegans* do not have appreciable levels of DNA methylation, yet do display inheritance of histone marks, raising the possibility that there is another mechanism for the transmission of histone modifications between generations (Greer and Shi, 2012).

Small RNAs, such as piRNAs, can be inherited trans-generationally (Brennecke et al., 2008; Ashe et al., 2012). The piRNA interacting protein PIWI has been implicated in epigenetic regulation (Yin and Lin, 2007) and is important for suppressing phenotypic variation. It has been hypothesized that PIWI may have a role in canalization of traits over evolutionary time (Gangaraju et al., 2011).

Other small RNAs can be passed through the germline to the developing embryo. Well known examples include small non-coding RNAs passed through the sperm that target the *Kit* locus in mice generating a white tail phenotype in the offspring (Rassoulzadegan et al., 2006) and the miR-1/Cdk9 paramutants that are associated with cardiac hypertrophy in mice (Wagner et al., 2008). Although the exact mechanism is unknown, a recent study has shown that the methyltransferase *Dnmt2*, previously thought to target only tRNAs, is required for this process (Kiani et al., 2013).

Population and Quantitative Epigenetics. The effects of epigenetic changes, and epialleles of genes, have not been extensively investigated in the fields of population and quantitative genetics (Geoghegan and Spencer, 2013a, 2013b). Recent evidence from comparing plants in different environmental or growth conditions has shown that genomes are capable of containing single-methylation-polymorphisms as well as single-nucleotide polymorphisms (Schmitz et al., 2013). These stable differences in DNA methylation, with no underlying change in the base-pair that is methylated, may have a significant role in determining individual and population fitness, particularly in response to fluctuating environments. There may also be evolutionary consequences, as variation in DNA methylation may act to mediate the adaptive value of a trait. These variants may also play a role in resolution of genomic conflicts, both with selfish genetic elements and intersexual conflict via imprinting (Johnson and Tricker, 2010).

We are yet to see large-scale population studies of epigenetic change in animal genomes, so we do not yet know if they have a significant impact on our understanding of population dynamics. Such studies have been hindered by the cell specific nature of epigenetic modifications and the lack of techniques that are fast and cheap enough to probe such modifications in multiple samples.

The Interaction Between Epigenetic and Genomic Variation. Single nucleotide polymorphisms (SNPs) are abundant in animal genomes. The most common polymorphism is a transition from a C to a T nucleotide. These polymorphisms can affect numerous CpG sites in the genome, by altering a C in a CpG dinucleotide to another nucleotide that cannot be methylated (Shoemaker

et al., 2010). Studies have shown that these SNPs can influence gene expression via effects on DNA methylation (Bell et al., 2011; Gutierrez-Arcelus et al., 2013). The effect of SNPs on DNA methylation can either be direct, by changing a C (in a CpG dinucleotide) to a non-modifiable nucleotide, or indirect by altering transcription factor binding, which in turn independently affects gene expression and DNA methylation levels (Gutierrez-Arcelus et al., 2013).

Polymorphisms may also affect imprinting locus control regions and thus have an influence on epigenetic changes associated with parental imprinting (Coolen et al., 2011). This concept of allele-specific methylation is growing in importance with the recognition that this phenomenon may extend well beyond classical imprinted genes.

Tools and Pitfalls of Epigenomic Techniques

The majority of the current methods to study DNA methylation rely on bisulphite conversion of the DNA (Laird, 2010). Treatment of the DNA with bisulphite causes unmethylated cytosines to be converted to uracils which, using common molecular techniques such as PCR and sequencing, are detected as thymines. Methylated cytosines are protected from conversion and are detected as cytosines. DNA methylation at individual loci can be interrogated using PCR and Sanger sequencing, high-resolution melting (Wojdacz and Dobrovic, 2007, 2009), MethyLight (Eads et al., 2000) and epiTYPER (reviewed in McLean et al., 2012). Next generation sequencing has meant that DNA methylation can now be interrogated on a genome wide scale by shot-gun sequencing (Cokus et al., 2008; Lister et al., 2009) or by reduced representation bisulfite sequencing (RRBS) (Chatterjee et al., 2012). DNA methylation can also be interrogated by restriction enzymes that target methylated DNA (i.e., Guo et al., 2011) and this method can also detect 5′ hydroxymethylcytosine (Davis and Vaisvila, 2011). Antibodies against 5′ methylcytosine and 5′ hydroxymethylcytosine can be used to enrich for methylated regions of the genome (m-DIP (Weber et al., 2005), or hmc-DIP (Davis and Vaisvila, 2011)) prior to next-generation sequencing or array analysis.

The "gold standard" method to study histone modifications involves using antibodies to a histone modification of interest (i.e., H3K27me3) to affinity purify fragments of chromatin (chromatin immunoprecipiatation or ChIP). The DNA is then eluted and analyzed by quantitative PCR to interrogate a single locus (ChIP-PCR) or array hybridization (ChIP-chip) or next-generation sequencing (ChIP-seq) for genome-wide data (reviewed in Furey, 2012). New approaches combine bisulfite sequencing of ChIP DNA to simultaneously detect DNA methylation associated with particular histone modifications (Brinkman et al., 2012; Statham et al., 2012).

Techniques for detecting higher-order chromatin structure within the nucleus focus on a technique known as chromatin conformation capture (3C) and its derivatives including 4C, 5C, 6C, Hi-C, and ChIA-PET (Sajan and Hawkins, 2012; Dekker et al., 2013).

General Limitations of Epigenomic Techniques. Many of the techniques described above require significant quantities of starting DNA or chromatin material. Obtaining this material may be difficult for small animals or tissues with limited cell numbers. These techniques have been established and used extensively in the analysis of cell cultures; their use in animal tissues is inherently more complex. There are hundreds of different cell types in humans (Vickaryous and Hall, 2006), all of which have cell-type specific gene expression and presumably epigenetic marks. Even within a single tissue such as the liver, multiple cell types are present. Indeed methylation profiles have been suggested as surrogates for characterizing cell mixtures (Houseman et al., 2012) Thus any analyses of most biospecimens gives an aggregate reading of epigenetic marks across the tissues or cell types. When applying these techniques in vivo it is important, where possible, to obtain a homogenous cell population. If working with model organisms it is possible to use fluorescent cell type specific reporters or antibodies together with fluorescence activated cell sorting (FACS) to obtain relatively homogenous cell populations (i.e., Berger et al., 2012; Harzer et al., 2013).

Such approaches generate large amounts of data, particularly when coupled with next-generation sequencing. The amount of data, and the fact that epigenetic marks and fixed genomic variation likely function in combinations, means that there is complexity in terms of data analysis and statistics. There are a number of specialized programs for analysis of whole-genome epigenetic data, particularly for DNA methylation and histone modifications, but there is no standardized way to analyze this data and each experiment may require a customized bioinformatics and statistical approach. A further issue is that epigenetic data has a number of intrinsic characteristics, some methodological, others relating to how it is expressed, that means it often deviates significantly from the normal distribution, and complex transformations are needed that can limit use of traditional statistical approaches (Wutz and Gribnau, 2007). The analyses described here can also be time-consuming and expensive, and it is important that these experiments are performed in a standardized way. This ensures the quality of the data, and more importantly, allows comparison of data generated from different laboratories in the same experimental system.

The likelihood of epigenetic mechanisms having an influence on the evolution of phenotypic plasticity and assimilation of acquired traits relies in part on transgenerational inheritance of epigenetic marks. There is evidence to support the transgenerational transmission of epigenetic information (Jablonka and Raz, 2009), but in general detecting incidences of this is complicated, particularly in species that develop in utero as the mother, embryo and the germ cells of the embryo (the future grandchildren) all share a common environment. This means that

at least three generations are required to confirm transgenerational epigenetic inheritance in females, and two in males (Jablonka and Raz, 2009). It is also important to distinguish between transgenerational transmission of information, parental transmission and so called "niche reconstruction" or indirect epigenetic inheritance, whereby similarities in the animals experience or environment influences epigenetic programming, causing similarities in the epigenetic marks between parents and offspring (Weaver et al., 2004; Champagne and Curley, 2008). While these parental effects are transmitted from generation to generation they are not mediated by trans-meiotic transmission of epigenetic marks.

Future Perspectives: How Do We Move From Phenomenon to Function?

We now have techniques that enable rapid assessment of different sorts of epigenetic variation. We need to begin to investigate the role and function of such variation, and of epigenetic changes in response to plasticity, in evolution. Here we discuss some of the major challenges that are currently hampering progress in this research field.

Finding mechanisms that linking environmental perturbation to epigenetic change. We have evidence to suggest that environmental cues can be transmitted to changes in the epigenetic regulation of the genome in many animals, and that these changes may be passed to future generations via the gamete or, at least for one to two generations, through parental effects. But in many cases we do not have a good understanding of how the environmental signals are transmitted to the affected cells. In insects environmental change is often linked to hormone titer, in particular juvenile hormone and ecdysteroids (Hartfelder and Engels, '98; Oostra et al., 2011; Ishikawa et al., 2012). However, it is not known if these molecules are the primary effectors of environmental change, and if so, how they might influence the epigenetic status of specific cells or cell types. Most work in mammals has focused on nutritional manipulation or glucocortiocoid-mediated effects mimicking stress.

Systematic analysis of molecules/signaling pathways that link environmental perturbations, such as temperature, nutrition or stress, with plastic events will allow us to not only understand how environmental signals are translated into epigenetic changes but also if, and how, these changes are transmitted to the germ-line from distant tissues. Direct transmission of epigenetic marks through the germ-line would provide a direct mechanism by which environmental perturbation might influence evolutionary processes. Further, undertaking such analyses in phylogenetically diverse animals will allow us to place these molecular mechanisms in an evolutionary context: do similar mechanisms relay environmental challenge in diverse species. Is then the transmission of environmental information to the epigenome an ancient and conserved feature of animals?

Linking epigenetic variation to phenotype. DNA methylation and modified histones are associated with numerous genomic features, including transposable elements, centromeres and transcribed genes. If we find variation in DNA methylation, or histone modifications around specific loci, does it have any consequence for the cell or the animal? It is possible that some, if not the majority, of epigenetic variation, viewed in a particular region of DNA sequence, have little or no functional consequence under normal conditions; they do not affect gene expression and therefore do not affect phenotype. It is also possible that these epigenetic variants may be functionally important in a different context, that is, under different cellular or environmental conditions. If we are to be able to understand the importance of epigenetic variation, we need first to understand if such changes have any functional effect at all. It is not enough to show that two individuals have a difference in methylation or chromatin modification, we have to show that that has some impact on those individual's phenotype. The recognition of allele-specific methylation raises the issue of whether a particular epiallele is acting independently of fixed genomic variation. Linking epigenetic variation with phenotype data firstly relies on accurate measurements of an appropriate phenotype. We do not yet have a good understanding of how the epigenome is affected by environmental conditions (see above), and in a complex environment an animal receives information about innumerable parameters that may affect the epigenome, and phenotype. But, in many cases, we are limited in the phenotypes that we measure and any epigenetic variant may only contribute subtly, perhaps negligibly, to an animal's phenotype, making it difficult to detect epigenetic variants associated with a phenotype. However, once variants have been identified the biggest challenge is determining whether the epigenetic variant is causative of the phenotype or simply correlated.

Manipulation. We need to separate causation from correlation. That epigenetic changes are correlated with a particular phenotype may just indicate that you are measuring the phenotype, and that these changes are a consequence, and not a cause, of the process you are interested in. Currently manipulation of epigenetic mechanisms is limited to whole-scale, broad range perturbation, that is by treating the animal or cells with inhibitors of DNA methylation. However, we need to be able to interrogate the function of DNA methylation or histone modifications at a specific site. Thus we need to be able to develop techniques to specifically target epigenetic modifications to particular places in a genome to determine if those changes are causative, or indeed even functional rather than simply correlated with a phenotype.

Cellular approaches. It is important to be careful to acknowledge the huge problem that the cell-type specific nature of epigenetic modifications poses for our understanding of their role in evolution, as well as their analysis. As stated previously,

epigenetic marks and changes are, on the whole, cell specific. If epigenetic changes are to be assayed in evolutionary studies, it is vital that it is done in specific cell types. Tools, such as fluorescence-activated cell sorting, laser capture microscopy or micro-dissection exist to do this, but they are rarely employed. Tissues with low cellular heterogeneity like buccal smears, may also avoid the averaging effect.

Future Perspectives: Evaluating the Role of Plasticity and Epigenetics in Evolution

The role of plasticity (West-Eberhard, 2003; Pigliucci et al., 2006; Crispo, 2007) and indeed epigenetics (Feinberg and Irizarry, 2010; Bateson and Gluckman, 2011) in driving evolutionary processes is an active area of research. Historically, it has been proposed that plasticity may affect evolutionary processes directly, in that the ability to alter phenotypes to accommodate a fluctuating environment means that plastic individuals within a species will be more likely to reproduce and, assuming that plasticity is encoded genetically, this will be heritable (Baldwin, '02). This will, over evolutionary time, result in stabilization of generalized plasticity within species, but not allow a specific plastic trait to become stabilized within a species. It is argued that, over evolutionary time, Darwinian selection will act on existing genetic variation for that trait to be genetically accommodated (West-Eberhard, 2003). This theory predicts that plasticity in species exposed to fluctuating environments will increase over evolutionary time, and that some plastic traits will become encoded by the genome if they are advantageous, and if genetic variation exists in the population to allow this. If this mechanism prevails then we might expect epigenetics and plasticity to have a limited affect on evolutionary processes. Recent studies indicate that there may be a more direct route from environmental perturbation to genetic accommodation; via transgenerational transmission of epigenetic information (Anway et al., 2005; reviewed in Jablonka and Raz, 2009; Stouder and Paoloni-Giacobino, 2010; Greer et al., 2011; Stouder and Paoloni-Giacobino, 2011; Ashe et al., 2012; Manikkam et al., 2012a). This mechanism may allow specific information about the environment to be passed by an animal to its offspring, prefiguring the next generation to be successful in a particular environment. Direct transmission of epigenetic information between generations increases the scope for evolutionary processes to be affected by epigenetic information and plasticity. Ultimately this also requires a mechanism for stable integration of this information into the genome (such as deamination of methylated cytosine residues (Feinberg and Irizarry, 2010; Bateson and Gluckman, 2011)). Understanding how, and with what frequency, environmental perturbation can influence evolutionary processes is critical to our understanding of plasticity and the integration of plasticity into evolutionary theory.

Summary

The ubiquity of epigenetic modification of the genome, its influence through the life-course and transgenerationally, and its environmental responsiveness, mean that epigenetic modifications are likely to have a significant impact in evolutionary studies. The advent of high throughput sequencing and biochemical techniques to measure modifications allows researchers to access the epigenome, and perhaps begin to understand the interface between epigenetic and evolution. There are pitfalls to these approaches, and only with knowledge of these, and the invention of techniques to manipulate epigenetic marks, will we be able to see clearly the influence of epigenetics on phenotype, plasticity and ultimately, evolution.

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