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Epigenetics in Insects: Genome Regulation and the Generation of Phenotypic Diversity

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Abstract

Epigenetic inheritance is fundamentally important to cellular differentiation and developmental plasticity. In this review, we provide an introduction to the field of molecular epigenetics in insects. Epigenetic information is passed across cell divisions through the methylation of DNA, the modification of histone proteins, and the activity of noncoding RNAs. Much of our knowledge of insect epigenetics has been gleaned from a few model species. However, more studies of epigenetic information in traditionally nonmodel taxa will help advance our understanding of the developmental and evolutionary significance of epigenetic inheritance in insects. To this end, we also provide a brief overview of techniques for profiling and perturbing individual facets of the epigenome. Doing so in diverse cellular, developmental, and taxonomic contexts will collectively help shed new light on how genome regulation results in the generation of diversity in insect form and function.

INTRODUCTION

Epigenetic information directs the formation of distinct cellular and organismal phenotypes from a common genome (19). For example, the ability of insects to develop phenotypes appropriate to their environment relies on epigenetic information (99, 136). Epigenetics is specifically concerned with heritable changes to gene regulation that occur in response to intercellular and extracellular environmental cues. Broadly defined, epigenetic information can take many forms, since factors at many levels can stably affect gene regulation. However, the field of molecular epigenetics is generally concerned with molecular mechanisms that directly affect, alter, or interact with chromatin.

Epigenetic information can be transmitted through mitotic cell division within individuals and meiotic cell division leading to the production of offspring. Intragenerational epigenetic inheritance is concerned with the process of development and addresses questions of how an egg with a single set of genetic instructions is able to develop into a multicellular organism made up of distinct tissues (138). In contrast, intergenerational epigenetic inheritance (54) is concerned with how a focal individual transmits epigenetic information to offspring. This distinction is important, and intergenerational epigenetics, though thought to be rare, is of interest because it directly affects the process of evolution. Nevertheless, this review focuses primarily on intragenerational epigenetic inheritance because the majority of research on molecular epigenetics in insects has been conducted at this scale.

The goal of this review is to provide a primer on epigenetic inheritance. We consider three major epigenetic inheritance systems: DNA methylation, histone modifications, and noncoding RNAs. Further, we provide a brief overview of techniques used to assay epigenetic information that are applicable in insects. We hope this information will prove useful to those who wish to delve into the study of epigenetic inheritance and help further our understanding of epigenetics in insects.

DNA METHYLATION

DNA methylation is perhaps the most faithfully heritable form of epigenetic information. Unlike other forms of epigenetic information, however, DNA methylation is essentially absent from the genomes of many insect groups. Each of six investigated insect orders exhibits at least one evolutionary loss of genomic DNA methylation, and there is no evidence for substantial DNA methylation in the genomes of investigated dipterans, including *Drosophila* (14, 149). In recent years, DNA methylation has nevertheless captured the attention of entomologists, driven in large part by a desire to understand the importance of DNA methylation to developmental plasticity.

Mediators and Inheritance of DNA Methylation

DNA methylation is mediated by two classes of enzymes in animals: de novo DNA methyltransferases (DNMT3 proteins) and maintenance DNA methyltransferases (DNMT1 proteins) (86). In animals, DNA methylation occurs primarily by the addition of a methyl group to cytosines residing in a 5′-cytosine-phosphate-guanine-3′ dinucleotide (CpG) context (86). The symmetrical nature of CpGs provides a mechanism to reestablish patterns of methylation after semiconservative DNA replication; DNMT1 restores symmetrical methylation by targeting hemimethylated CpGs (86). Cytosine methylation has also been reported in a non-CpG context in insect genomes but at much lower levels than CpG methylation (17). Therefore, we use the term DNA methylation synonymously with cytosine methylation in a CpG context.

Our understanding of the molecular functionality of DNA methyltransferases in insects is informed by homology to well-studied mammalian orthologs and, thus far, a limited number of experiments in insect taxa (98, 141). Notably, several insect taxa that lack DNMT3 nevertheless

exhibit DNA methylation (14, 143). This suggests that DNMT1 has either attained some level of de novo methylation functionality or that maintenance methylation is sufficient to maintain DNA methylomes over evolutionary time. The latter hypothesis posits that extensive DNA demethylation and reprogramming, which occurs between generations in mammals (93), does not occur in insects. Indeed, study of DNA methylation in two species of *Nasonia* wasps revealed that species-specific patterns of DNA methylation were retained in the parental alleles of F1 hybrids (139). This finding is consistent with the hypothesis that DNMT1 faithfully transmits intergenerational DNA methylation, but further research is warranted on this topic.

A major area of interest in terms of intergenerational DNA methylation is its potential role in genomic imprinting, the process by which variation in gene expression is shaped by an allele's parent of origin (39). A role for DNA methylation in imprinting has been observed in plants and mammals (70, 78). Moreover, imprinting is predicted to arise in response to evolutionary conflicts between relatives, which are pronounced in eusocial insects (110). In line with these predictions, recent evidence for parent of origin effects on transcription has emerged in the social bee *Apis mellifera* (42). However, robust evidence for parent of origin effects caused by DNA methylation acting as a mediator of imprinting in insects remains elusive (113). In particular, although there seems to be evidence that gene expression patterns are sometimes heritable, additional experimental analyses will be required to demonstrate conclusively that DNA methylation is the epigenetic cause of such heritable effects.

Genomic Patterns of DNA Methylation

Many vertebrates exhibit global patterns of genomic DNA methylation, wherein the vast majority of CpGs in both intragenic and intergenic regions are methylated (131). In contrast, the genomes of invertebrates possess relatively sparse patterns of DNA methylation (**Figure 1**) (131). In insects, methylation is preferentially targeted to genes that are broadly expressed across tissues and organismal morphs (41, 45, 47). Within these genes, DNA methylation is highly enriched in exon sequences of holometabolous insects, as documented in Lepidoptera (62, 143), Coleoptera (32), and Hymenoptera (45). In contrast, DNA methylation occurs in a much larger proportion of the genome in hemimetabolous insects, as observed in Blattodea (14, 47), Hemiptera (14), and Orthoptera (140). For example, DNA methylation is enriched in gene-dense regions in the termite *Zootermopsis nevadensis* but is prevalent in introns as well as exons (47). Interestingly, there is currently no robust evidence for DNA methylation enrichment in transposable elements or gene promoters of insect genomes (47).

The mechanisms mediating recruitment of de novo DNA methyltransferases are poorly understood. However, genomic profiling of DNA methyltransferases in mammals revealed that DNMT3B is associated with the methylation of gene bodies (11), where DNA methylation is most prominent in insect genomes. In mammals, DNMT3B methylates DNA associated with nucleosomes that bear modifications typical of transcribed genes (11, 102). A similar mechanism, whereby DNMT3 is recruited according to patterns of covalent histone modification, may operate in insect taxa, but this hypothesis remains speculative (49).

Molecular Function of DNA Methylation

Associations between DNA methylation and transcriptional regulation depend upon genomic context (64). Nevertheless, a general effect of DNA methylation on transcription factor binding has been observed. The DNA binding specificity of a majority of 542 transcription factors in human cell lines are altered by variation in DNA methylation, with methylated cytosines either increasing or decreasing binding of a specific transcription factor (147). These results support a

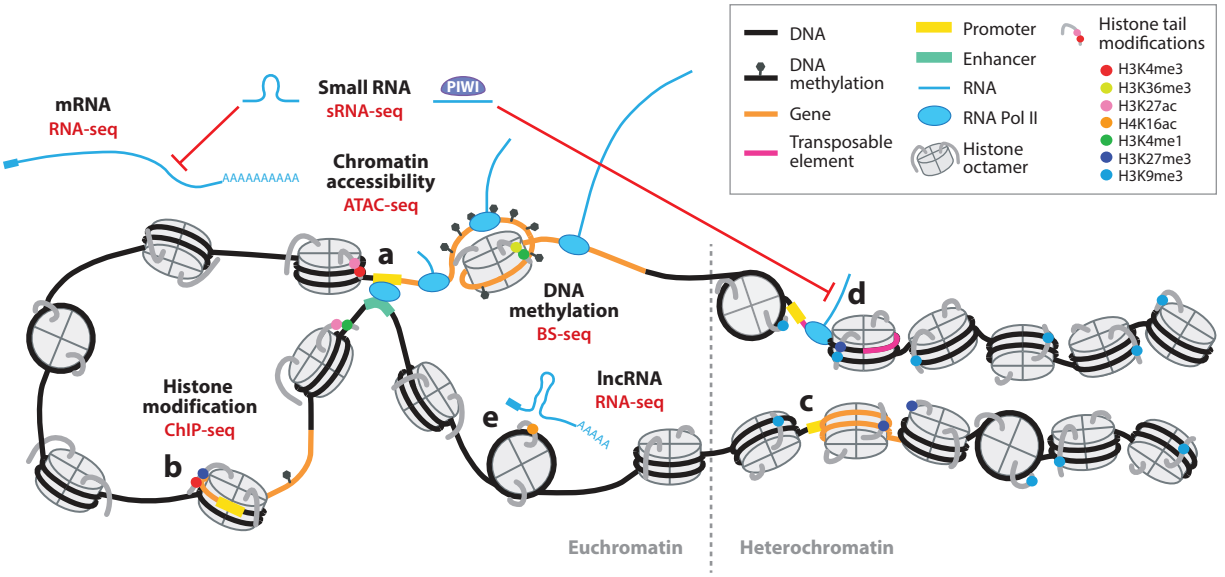

Figure 1

Key molecular features of the insect epigenome. Genes that are (*a*) actively expressed, (*b*) bivalent and poised for expression, and (*c*) stably repressed are depicted with characteristic histone modification and DNA methylation. In addition, (*d*) a transposable element silenced by PIWI-interacting RNA (piRNA) regulation and (*e*) a long noncoding RNA (lncRNA)-mediated histone modification are shown. Red text denotes common sequencing methodologies used to profile the epigenome. Abbreviations: ATAC-seq, assay for transposase-accessible chromatin using sequencing; BS-seq, bisulfite sequencing; ChIP-seq, chromatin immunoprecipitation followed by sequencing; mRNA, messenger RNA; Pol II, polymerase II; sRNA, small RNA.

general mechanism that may explain many of the gene regulatory effects of DNA methylation observed across eukaryotic taxa.

DNA methylation in regulatory elements can influence gene expression levels by affecting transcription factor binding and recruitment of the transcription initiation complex. However, the functional significance of DNA methylation within gene bodies, as is most frequently observed in insects, is less clear. A recent study of mice revealed that intragenic DNA methylation may enhance transcriptional fidelity by reducing transcript initiation at improper sites, a process that may, again, be mediated by transcription factor binding (102). Interestingly, the most sparse insect DNA methylomes profiled to date have retained DNA methylation within constitutively expressed gene bodies in regions near, but downstream of, transcription start sites (**Figure 1*a***) (32, 45, 62). This finding is consistent with the hypothesis that DNA methylation helps to partition promoter regions from regions of transcriptional elongation in actively expressed genes (46, 62). The effects of DNA methylation on transcription factor binding may also influence patterns of transcript splicing because transcription factor presence within the gene body can slow RNA polymerase II (RNA Pol II) processivity and enhance the recognition of weak splice sites (77, 122).

Phenotypic Plasticity and DNA Methylation

Interest in a role for DNA methylation in insects surged after an experiment reduced expression of *dnmt3* in honey bee larvae and led to a dramatic shift from worker to queen developmental fate (71). Subsequent research revealed the presence of differential DNA methylation in the brains of adult honey bee queens as compared to workers (87) and in heads of queen-destined versus worker-destined larvae (40). However, questions were raised about the nature of differential DNA

methylation detection in these and similar studies in ants (17, 48) because of concerns over sufficient biological replication (79). Indeed, a study of the clonal raider ant revealed no significant differences in DNA methylation in replicated brain samples of distinct worker phenotypes (79), and a well-replicated study of the honey bee revealed no significant differences in DNA methylation in brains of newly eclosed queens and workers (56).

However, the same study that identified no differential methylation between brains of newly eclosed queens and workers in the honey bee did, in fact, identify 155 differentially methylated regions in replicated samples of brains of workers taken before and after a behavioral transition from nursing to foraging (56). Remarkably, reversion from foraging back to nursing was accompanied by reversions in DNA methylation for around one-third of these loci (56), suggesting a dynamic association between DNA methylation and transcriptional activity. Moreover, a study of whole bodies of distinct castes and sexes of the termite *Zootermopsis nevadensis* identified 2,720 differentially methylated genes among different termite morphs in a biologically replicated experiment (47). The high number of differentially methylated genes in this study are, in part, likely reflective of differences in the tissue composition of distinct castes and the relatively high levels of DNA methylation in *Z. nevadensis*. Regardless, these results point to DNA methylation differences arising during postembryonic development. Overall, more research needs to be undertaken to determine whether such differences in DNA methylation between phenotypes are common in insects and whether they have meaningful functional consequences.

MODIFICATIONS OF CHROMATIN-ASSOCIATED PROTEINS

The majority of DNA in the metazoan nucleus is incorporated into nucleosomes, which are composed of approximately 147 bp of DNA wrapped around a protein complex composed of eight histone proteins (132). These proteins are arranged in two tetramers, each containing histones H2A, H2B, H3, and H4. Histone proteins are fundamentally important to organismal function and are some of the most conserved proteins in eukaryotes (12, 151).

DNA bound to nucleosomes is much less accessible to other proteins. For example, transcription factors typically bind only to sites lacking nucleosomes (12, 55). Therefore, many regulatory processes in eukaryotes have been linked to the alteration of histone–DNA interaction, and many other proteins bind to histones (6, 151).

There are several important ways in which nucleosomes can be altered to impact the regulation of genes. First, a histone protein may be modified after translation. Second, an alternative sequence variant of a histone may be substituted for its core histone equivalent. Finally, a nucleosome can be physically moved to reveal important underlying binding sequences. There is strong evidence that nucleosomes and their modifications are heritable across cell divisions. Therefore, histone modification and replacement are both important epigenetic modifications across eukaryotes.

Histone proteins have been studied extensively in *Drosophila*, but only recently have histones been investigated in nonmodel insect taxa (76, 121, 123, 124). Histone protein modifications are numerous, functionally conserved, and have been strongly linked to multiple forms of gene regulation in diverse taxa (68). Thus, histone proteins may be more directly implicated in the mediation of phenotypic plasticity than DNA methylation (79, 123, 124).

Covalent Histone Modifications

Histone posttranslational modifications (hPTMs) are a diverse set of epigenetic signals (151) that typically occur on histone protein N-terminal amino acid tails. There are several ways in which hPTMs can alter transcription. First, the association between the target histone and underlying DNA can be directly impacted by the addition of a chemical group to a histone protein (107),

which may consequently increase or decrease the ability of transcription factors to access DNA. Second, many proteins bind histone tails, often contingent upon the presence of hPTMs. The modification of histone residues can thus be translated into specific functional outcomes through the actions of effector proteins (6, 50).

There are numerous proteins that modify histones (8, 91). In some cases, modifier proteins may alter several residues, while in other cases, distinct residues require distinct transferases for their modification (127). Modifier proteins are localized to their target regions by binding to other engaged proteins, recognizing specific signatures of chromatin, or direct binding to specific DNA sequences (6, 50).

Common hPTMs include the addition of acetyl, methyl, or phosphorus groups, and the most commonly modified residues of histone tails are lysine and arginine (151). Lysine acetylation is perhaps the most well-studied modification. The addition of acetyl groups to lysines of the histone tail can greatly reduce the histone's electrostatic attraction to negatively charged DNA. This increases the accessibility of underlying DNA or facilitates the movement of the nucleosome along the DNA, both of which allow for the binding of DNA-binding factors (36, 150, 151). Consequently, actively expressed gene promoters and active distal enhancer elements are typically enriched for acetylation at multiple lysines on the tails of histones (**Figure 1*a***) (36, 128).

Histone acetylation represents an example of how hPTMs may directly impact the strength of the nucleosome's histone–DNA association. However, other hPTMs are instead recognized by specific proteins that alter transcription. For example, histone methylation is not thought to directly facilitate the physical opening of chromatin (150) but instead represents information that is interpreted by reader proteins (50). In this way, methylation of different histone residues can have differing outcomes depending upon the specific hPTMs and the proteins reading them (114). For example, H3K9me3 is associated with heterochromatin repression, while H3K4me3 is associated with activated transcription (**Figure 1*a*,*d***) (151).

The complex way in which hPTMs are targeted and deposited can be illustrated by the hPTMs H3K4me3 and H3K36me3, which are both associated with actively expressed genes. Within active genes, these marks have distinct spatial localization: H3K4me3 is largely confined to promoter regions, whereas H3K36me3 is targeted to gene bodies (**Figure 1*a***) (151). These alternative patterns arise because the form of RNA Pol II associated with transcriptional initiation is bound by the methyltransferase responsible for establishing H3K4me3, whereas the form of RNA Pol II associated with transcriptional elongation is bound by the methyltransferase responsible for H3K36me3 deposition (22).

The behavior of H3K4me3 also illustrates another characteristic of hPTMs: their combinatorial nature in directing regulatory outcomes. While H3K4me3 marks the promoters of actively transcribed genes, it is also present in the promoters of some genes that are not actively expressed. However, these latter genes lack H3K36me3 and several other promoter acetyl modifications, and instead possess H3K27me3, a modification typically associated with repressed or transcriptionally inactive chromatin. In combination, these two modifications are associated with genes that are not expressed but instead are "poised" for activation upon induction (**Figure 1*b***) (61, 151).

Importantly, information stored in nucleosomal hPTMs can be transmitted across cell divisions (8, 91). Emerging evidence suggests some modifications may be maintained through reincorporation of parental histones into newly synthesized DNA during DNA replication (3) (but see 2). Cells may also use hPTM information on existing histone proteins or integrate *trans*-acting factors to help perpetuate parent hPTM patterns during or after DNA replication (91).

Few studies have examined hPTMs in depth in insects outside of *Drosophila*, but these non-*Drosophila* studies reveal the promise of studying hPTMs. For example, hPTMs are tightly linked to transcriptional variation associated with behavioral transitions in ants (123, 124), and hPTMs show

stage-specific variation associated with head development in lepidopterans (76). Thus, hPTMs offer a promising avenue of research for studies of insect epigenetics.

Histone Variants

Replacement of canonical histone proteins with sequence variants can considerably alter gene expression (132). Variants of canonical histone proteins can contain unique residues that are subject to novel modifications or simply variant-specific sequence motifs that are bound by specific effectors (7). Several key histone variants are conserved between insects and mammals and are also linked to gene regulation in *Drosophila*. Therefore, histone variants are promising candidates for study in other insects.

The histone H2A.v (H2A.Z in vertebrates) is a variant of the canonical histone H2A (7, 132). Nucleosomes containing H2A.v possess different characteristics than those without it (7). Specifically, H2A variants increase the mobility of, or destabilize, the nucleosomes in which they are found, resulting in increased accessibility of DNA (132). Thus H2A.v is typically associated with active gene promoters. In contrast, the histone variant H3.3, which has been well studied in *Drosophila*, is typically found in the bodies of active genes and differs from its parent histone, H3, by only a few amino acids. Most canonical histones are incorporated into DNA during cell division. However, the incorporation of H3.3 is replication independent (1). Importantly, actively transcribed genes are associated with nucleosome eviction, which likely explains the preferential incorporation of H3.3 into the chromatin of actively expressed genes (72). Nevertheless, the direct functional consequences of H3.3 incorporation are currently unclear, as H3.3 has also been observed in heterochromatic regions (97, 132).

NONCODING RNAs

Much of the metazoan genome is transcribed despite the fact that only a fraction of the genome consists of protein-coding genes. Noncoding RNAs (ncRNAs) are a heterogeneous class of RNAs that are not translated into proteins. While some of these RNA products may have no specific function, some ncRNAs play a role in regulating cellular processes (27, 58, 90). Four types of ncRNAs have been implicated as potentially having epigenetic effects: PIWI-interacting RNAs (piRNAs), microRNAs (miRNAs), small interfering RNAs (siRNAs), and long noncoding RNAs (lncRNAs). However, the evidence that piRNAs and lncRNAs have epigenetic effects is particularly strong in insects (28), and so these are given special attention here.

PIWI-Interacting RNA

piRNAs are highly variable, short (21–35 nucleotides), single-stranded ncRNAs that can initiate epigenetic effects within insect genomes. piRNAs were first identified in *D. melanogaster*. Therefore, most of what is known about the molecular basis of piRNA function is derived from experiments in *Drosophila* (28, 67). piRNAs apparently evolved in response to the detrimental effects of transposable elements (TEs) (67). TEs are ubiquitous in insect genomes (92) and can cause damage through aberrant recombination events and deleterious mutations. piRNAs act to silence TEs and therefore prevent harmful TE-induced mutations (57, 67). Interestingly, new types of piRNAs may be derived from invading TEs and then incorporated permanently into the genome (57). In this way, the piRNA system may act as a type of inherited immunity (67, 116).

piRNAs are involved in at least two types of epigenetic effects (28, 33). First, the PIWI complex can promote the generation of heterochromatic marks where TEs are found (28, 95, 115). piRNAs

apparently recruit cofactors that lead to histone modification (H3K9me3), accumulation of the heterochromatin protein HP1a, an increase in the density of the linker histone H1, and exclusion of RNA Pol II (58, 60). Thus piRNAs interact with transcribed genes to induce cotranscriptional silencing (**Figure 1*d***).

Second, some piRNAs are maternally transmitted to offspring. The maternal cache of piRNAs acts to jumpstart the generation of piRNAs in developing embryos (28, 58, 67). The transfer of piRNAs from parent to offspring suggests that piRNA-mediated chromatin states can be inherited across generations (85). The information stored by this initial cache of piRNAs would allow for the reestablishment of chromatin states after meiosis (58, 85).

piRNAs and the associated PIWI proteins are present in many insects (28). However, there have been changes in gene copy number of the key PIWI proteins among insect taxa (28, 37). In addition, piRNAs showed biased expression patterns that have been interpreted as suggesting that they have functions besides TE suppression. For example, the PIWI pathway is apparently involved in processing viral RNA into piRNAs in mosquitos (4, 137). Finally, a particularly notable example of the diverse function of piRNAs in non-*Drosophila* species has been found in *Bombyx*, where piRNAs are directly associated with sex determination (69). Thus, diverse phenotypic consequences of piRNA regulation have been observed across insects.

microRNA

miRNAs are one of the best-studied classes of small ncRNAs. There are hundreds or thousands of miRNA genes in most species (52), and the biogenesis of miRNAs in insects seems to be fairly similar to that in noninsect taxa (84). miRNAs have been implicated in affecting a variety of behavioral, developmental, and physiological processes in insects (5, 13, 108). There is good evidence that miRNAs show epigenetic properties in plants and, perhaps, animals (112). However, the molecular evidence that miRNAs influence chromatin or are passed down through cell divisions in a truly epigenetic manner is still largely lacking in insects (28, 80). Therefore, the question of whether miRNAs affect chromatin and stably affect heritable information in insects requires further investigation.

Small Interfering RNA

siRNAs are similar to miRNAs in that they interact with proteins from the same families and participate in the RNA interference (RNAi) pathway. However, miRNAs are generally derived from the genome of the focal organism itself, whereas siRNAs can arise from several different origins, including viruses and TEs. There is evidence that siRNAs can directly influence chromatin states in several animals (80, 112) and therefore may have true epigenetic properties. In this case, siRNAs are loaded into the RNA-induced transcriptional silencing complex (RITSC) (26). The RITSC then binds to the specific genomic target and permits heterochromatin formation by recruiting chromatin-modifying enzymes to the target sequence. There may also be feedback interactions between siRNA production and different chromatin states in some eukaryotes (25, 58).

The siRNA pathway is found throughout insects (37) and has been particularly well characterized in *Drosophila* (90, 101). There is some evidence that the siRNA pathway may affect chromatin in *Drosophila* (44). It is believed that such modifications may occur when siRNAs become associated with nascent transcripts and the accompanying machinery helps recruit proteins that modify histones. For example, siRNAs may affect X chromosome inactivation in *Drosophila* (95). Thus siRNAs may be associated with the transmission of epigenetic information in insects (26).

Long Noncoding RNA

lncRNAs represent a broad category of RNAs that are over 200 nucleotides long and not translated into proteins (18, 38). lncRNAs can influence the function of genes in several ways (38, 43, 73). Some lncRNAs may act as facilitators of epigenetic regulation (53, 96, 100). For example, lncRNAs can bind to specific targets and recruit chromatin-modifying enzymes, initiating the formation of a silent or active chromatin state (**Figure 1*e***) (18, 38, 58, 95).

Perhaps the best-studied example of lncRNA function in insects is that of *roX1* and *roX2* genes, which are associated with X chromosome activity in male *Drosophila* (75). The dosage compensation complex (DCC) acts to modify the chromatin of the male X chromosome, leading to increased expression of male X-linked genes (74). Incorporation of the roX RNAs into the X chromosome allows the DCC to correctly target the X chromosome (30, 75). Ultimately, the DCC leads to modifications of the histone proteins on the male X that are associated with increased gene activity (30). *roX1* and *roX2* orthologs are found in several *Drosophila* species, and so this system of sex determination, and the associated lncRNAs, may be relatively conserved (111).

Although the best-studied insect lncRNAs are found in *D. melanogaster*, there has been considerable interest in the function of lncRNAs in other insect species. For example, several investigations have suggested that lncRNAs may be important to the function of honey bees (63, 88) and may be specifically involved in caste development (16, 146). Putative lncRNAs have also been identified in several other insects (142). Insect lncRNAs seem to evolve relatively rapidly, possibly indicating a general lack of functional constraint (82). Understanding whether putative lncRNAs are truly operational and functional products in non-*Drosophila* insects awaits experimental validation.

METHODS FOR ASSAYING EPIGENETIC INFORMATION

The recent explosion of new sequencing techniques paired with the rapid decrease in sequencing costs means that several techniques for assessing epigenetic information in insect genomes are becoming accessible to researchers in nonmodel systems. Here, we review several techniques for assaying epigenetic information genome-wide that can be applied in nonmodel taxa.

Assaying DNA-Associated Proteins

One of the more prevalent techniques for assessing DNA-associated proteins is chromatin immunoprecipitation followed by sequencing (ChIP-seq). ChIP-seq relies on antibodies to immunoprecipitate a DNA-associated protein target along with the bound DNA. The immunoprecipitated DNA can then be sequenced to reveal regions of the genome enriched for binding by the target protein (104). ChIP-seq has recently successfully been applied to insects beyond *Drosophila* (76, 123, 124, 126).

ChIP-seq has several limitations that, until recently, have made it challenging for application in nonmodel insects. For example, the cross-linking and sonication of conventional ChIP are harsh processes that decrease the availability of target molecules. Further, only a limited proportion of the genome may actually be bound by the target protein. Consequently, standard ChIP-seq preparations have low efficiency, which is particularly problematic for studies using already-limiting insect tissues. However, recent advances in library preparation are increasing the efficiency of ChIP-seq (118, 130). For example, ChIP-seq was successfully applied to single ant brains by using linear DNA amplification to attain sufficient material for analysis (123).

A technical advance that further facilitates working with limited material is native-ChIP (NChIP). NChIP-seq, an alternative to conventional ChIP-seq, utilizes micrococcal nuclease

to digest unbound DNA, which is followed by immunoprecipitation (103). The considerable gain in recovery afforded by NChIP means that very small amounts of tissue can be analyzed (21), allowing for the study of hPTM profiles in a single tissue of individual insects. For example, NChIP has been used to study *cis*-regulatory evolution among lepidopterans (76), as well as to study *Drosophila* tissues (83).

Nevertheless, NChIP does have limitations. For example, for DNA to be immunoprecipitated without cross-linking, the target protein must be strongly bound to the underlying DNA. This means that NChIP works best with proteins that are very tightly bound to DNA (but see 66). Another limitation is that proteins bound to DNA are not "frozen" at the moment of cross-linking as with conventional ChIP. In theory, this leads to a less precise mapping of protein–DNA interactions.

Finally, all ChIP-seq methods require the use of an antibody that specifically targets the protein of interest. Therefore, either proteins targeted by ChIP-seq must be conserved enough to allow the use of an antibody developed for use in another organism or novel antibodies must be developed in the target system. Antibody development can be prohibitive, as it is a costly and intensive process. Therefore, ChIP-seq in nonmodel organisms often targets proteins that are highly conserved. Conveniently, histones are some of the most conserved proteins in animal genomes (89), allowing the use of antibodies developed in *Drosophila*, or even mammals, for profiling hPTMs in nonmodel insects.

The assay for transposase-accessible chromatin using sequencing (ATAC-seq) has been employed to effectively profile regions of accessible (open) chromatin, as well as nucleosome positioning and transcription factor binding (15, 24, 35). Briefly, ATAC-seq utilizes a modified transposase to integrate sequencing adapters into regions of increased DNA accessibility. Thus, ATAC-seq ultimately produces sequences that correspond to open chromatin in the genome. Because adapter sequences can be integrated into accessible nucleosome-flanking DNA, as well as completely open regions, ATAC-seq data can be used to analyze DNA accessibility, as well as nucleosome positioning and transcription factor binding (24, 31). This method is relatively efficient, so reasonable sequencing libraries can be produced from as few as 500–50,000 cells in a single day (24, 31). Thus, ATAC-seq is an appealing method because it efficiently summarizes the epigenome by assaying DNA accessibility.

ATAC-seq suffers from a few constraints, however. Starting material should be fresh or cryopreserved (but see 31), and ATAC-seq works best with a single-cell suspension. Nevertheless, given the minimal input requirements and ease of execution, ATAC-seq is a prime candidate for profiling open chromatin in nonmodel organisms and has already been employed extensively in *Drosophila* (15).

Assaying DNA Methylation

Several techniques currently exist for assaying DNA methylation. One family of methods is based upon the action of sets of isoschizomer restriction enzymes that cut at the same CpG-containing motif but that are either methylation sensitive or methylation insensitive with respect to their digestion activity. These restriction digests can then be compared via electrophoresis, polymerase chain reaction (PCR) amplification, or quantitative PCR to infer coarse-level DNA methylation levels. Multiple variants of this core approach exist, including adaptations of amplified length polymorphism methods and pairing with bisulfite conversion.

The advantages to these techniques are the low cost and ease of implementation. However, several limitations exist. First, the specificity of whole-genome digestion methods is quite limited, or in the case of locus-specific methods, sensitivity is limited. Second, the interpretation of the

results of these restriction-based methods can be unclear. Thus, careful controls are essential when using restriction enzyme–based methods to assay DNA methylation, and even then, conclusions should be treated with caution and ideally confirmed by additional methodology.

At present, whole-genome bisulfite sequencing (BS-seq) is the most useful method for assaying DNA methylation (109). Briefly, this technique analyzes DNA that has been treated with bisulfite, which converts unmethylated cytosines to uracil and leaves methylated cytosines intact. The bisulfite-treated DNA is then PCR amplified, which effectively converts uracil to thymine, and sequenced. Thus, upon alignment to a reference genome, this method can be used to assess the sample's proportion of methylated cytosines at every site in the genome.

The primary limitation of this approach is that treatment of DNA with bisulfite is a harsh process that results in loss of up to 90% of starting genomic DNA. This means that BS-seq of specific insect tissues can be challenging. Secondly, incomplete conversion of DNA by bisulfite will result in false positives. This problem is addressed by inclusion of unmethylated spike-in DNA prior to bisulfite treatment. Finally, because all unmethylated cytosines are converted to thymines, genomic alignment of resulting sequencing reads is more challenging than conventional DNA sequencing. These problems are being overcome by ever-increasing read lengths and improved sequencing library preparation workflows. However, we expect BS-seq will ultimately be supplanted by a direct sequencing method that reliably and accurately differentiates methylated and unmethylated cytosines.

Methylated DNA immunoprecipitation (MeDIP) is another method for assessing DNA methylation globally. MeDIP is similar to ChIP. However, instead of using an antibody raised against a specific protein of interest, an antibody that specifically targets methyl-cytosine is used. When paired with high-throughput sequencing, MeDIP provides a genome-wide view of DNA methylation enrichment.

Assaying Noncoding RNA

Small RNAs, such as piRNAs, siRNAs, and miRNAs, can be readily assayed in insect genomes. In particular, total RNA can be extracted from tissues and then size selected for small RNAs only. Sequencing of these small RNAs (sRNA-seq) is followed by computational analyses to identify putatively functional RNAs. For example, miRNAs or pre-miRNAs may be identified on the basis of their inferred secondary structures and splice sites, and piRNAs show putative signals such as specific sequence motifs (81).

The properties of lncRNAs are similar to those of messenger RNAs (mRNAs) (135). For example, lncRNAs have typical 5′ caps and poly-A tails. lncRNAs can therefore be sequenced using conventional RNA-seq technologies. However, detecting low-frequency lncRNAs may require considerable sequencing depth. Regardless, a main difficulty in lncRNA analysis is distinguishing these molecules from standard protein-coding RNAs. Several computational methods have been developed to distinguish lncRNAs from coding RNAs (59), some of which rely on sequence analysis only. The alternative, using experimental approaches (translational tests or mass spectrometry), results in more confident classification of lncRNAs but is considerably more prohibitive in terms of cost and effort.

EXPERIMENTAL MANIPULATION OF EPIGENETIC INFORMATION

Assaying epigenetic information is a powerful technique for understanding the nature of the epigenome. However, hypothesis testing often requires more than genome-wide profiles. Consequently, methods for the manipulation of epigenetic information in nonmodel organisms are an increasingly important requirement of many investigations.

Drugs Affecting Epigenetic Information

One way to modify epigenetic information in insects is through the use of drugs that interfere with the deposition or removal of epigenetic marks. For example, several drugs exist that perturb patterns of DNA methylation, including 5-azacitidine and zebularine (29, 65). Some compounds also semiselectively inhibit the modification of histone proteins and have been used to inhibit the deposition or removal of acetyl groups (23) and/or the deposition or removal of methyl marks (133). Generally, these drugs block histone acetylases, deacetylases, methylases, or demethylases. For example, trichostatin A (TSA) is a selective inhibitor of class I and II histone deacetylases and has recently been used to alter the behavior of *Camponotus floridanus* workers in conjunction with an alteration of histone acetylation in their brains (123). Furthermore, one drug can be used to "rescue" the effects of another. For instance, the behavioral effects of TSA administration were reversed through the coadministration of C646, a drug that inhibits CBP [cyclic adenosine monophosphate response element–binding protein (CREB) binding protein], an important histone acetylase whose targets overlap those of TSA (123).

Drugs that affect epigenetic information do, however, have several limitations. For one, effects of drugs are often transient. While inhibition of a key epigenetic modification at a precise timepoint may have long-lasting effects on the organism, in cases where long-term alteration of hPTMs is required, drugs may be less useful. Secondly, many drugs have off-target effects. For example, decitabine, a popular drug used to inhibit DNMT activity, acts through its incorporation into genomic DNA, wherein it irreversibly binds to DNMTs. Thus, studies that use this drug are likely to suffer from off-target effects simply owing to DNA damage and other issues related to the irreversible linking of DNMTs with genomic DNA. Another issue inherent to the use of drugs that target histone-modifying enzymes is that many histone-modifying enzymes have roles in the modification of nonhistone proteins in the cell. For example, TSA administration can have effects on acetylation of many nonhistone proteins (125) and can have other off-target effects (20, 144), as can many other drugs that target histone-modifying enzymes (34).

Experimental Manipulation of Epigenetic Mediators

A different approach to perturbing the epigenome is to interfere with specific mediators of a given epigenetic mark using customizable reverse genetics methodology. RNAi involves the introduction of small RNAs that are incorporated into the RNA-induced silencing complex. This complex then targets mRNAs on the basis of sequence complementarity. The focal mRNA may then be degraded, or translation of the protein otherwise repressed. Consequently, highly specific interference with mRNA occurs, allowing for partial knockdown of a target transcript.

Despite its widespread use, RNAi has several weaknesses. For one, RNAi is transient, often lasting for 3–20 days after administration (9). RNAi can also have dramatically different effects on the basis of factors including administration route, siRNA design, and coadministration of a transfection reagent (105, 106). Nevertheless, RNAi has been an important technique used for knocking down the activities of genes in many insect systems (26, 51, 119, 148).

The CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats–CRISPR associated protein 9) system utilizes a microbial RNA-guided DNA endonuclease (Cas9) to make targeted deletions in an organism's genome (129). Along with the Cas9 protein, small guide RNAs (sgRNAs) are introduced into the cells of a given organism, facilitating targeted mutations on the basis of sgRNA complementarity. CRISPR has been used extensively in *Drosophila* (10), and three recent studies show the use of this system to create mutants in ants (134, 145) and lepidopterans (94). Recently, this system has been modified through the use of a catalytically inactive Cas9

(dCas9) to effect activation or repression of target genes (120). Thus, CRISPR-Cas9 can also accomplish nonmutational alteration of target gene expression, making it directly applicable to epigenomic studies.

Despite the potential advantages of CRISPR-Cas9, several issues may still arise from its use in insect systems. For one, CRISPR-Cas9 can result in off-target mutations (117). Furthermore, CRISPR-Cas9 still requires in-lab mating to produce homozygous mutants (although mosaic or heterozygous knockouts can be generated without mating). Nevertheless, CRISPR-Cas9 may be a manageable technique for those working with nonmodel insects who still wish to attempt transgenic manipulation of epigenetic function.

FUTURE ISSUES

The next several years should see a surge in studies of epigenetics in insects. Here, we suggest research directions deserving of attention in such efforts. Overall, profiling and perturbing individual facets of the epigenome in diverse cellular, developmental, and taxonomic contexts will collectively help to shed new light on gene regulatory mechanisms important to diversity in insect form and function.

1. The relationship between DNA methylation and phenotypic plasticity in insects remains unclear. An ideal investigation would couple experimental interference of DNA methyltransferase genes with nucleotide resolution profiles of genome-wide DNA methylation, transcriptome sequencing, and phenotypic screens. This would facilitate an evaluation of how DNA methylation affects organismal phenotype, as well as a comparison of the molecular effects of DNMT knockdown to gene regulatory differences arising from naturally occurring phenotypic plasticity.
2. The importance of hPTMs to the generation of phenotypic diversity is understudied and largely informed by model organism biology. Profiling key hPTMs in relation to phenotypic plasticity in multiple insect taxa is a necessary step to testing the generality of individual hPTM associations with gene regulation in insects. Studies of candidate hPTMs for developmental differentiation would ideally be followed by experimental perturbation of molecular mediators, epigenome profiling, and phenotypic screens.
3. The identification of ncRNAs in diverse insect systems will provide useful insight into the nature of these enigmatic molecules. Exploratory profiling should be coupled with experimental and evolutionary analyses to determine the function of specific ncRNAs within insect genomes.
4. Studies of intergenerational epigenetic inheritance should be undertaken to establish what epigenetic information is passed through generations (and what information is not). Such endeavors will lay the groundwork for addressing the mechanisms involved in putative genomic imprinting in insects.

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Errata

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