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SYMPOSIUM

The Function of Intragenic DNA Methylation: Insights from Insect Epigenomes

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Synopsis Epigenetic inheritance plays a fundamentally important role in mediating gene regulation and phenotypic plasticity. DNA methylation, in particular, has been the focus of many recent studies aimed at understanding the function of epigenetic information in insects. An understanding of DNA methylation, however, requires knowledge of its context in relation to other epigenetic modifications. Here, we review recent insights into the localization of DNA methylation in insect genomes and further discuss the functional significance of these insights in the context of the greater eukaryotic epigenome. In particular, we highlight the complementarity of the eukaryotic epigenetic landscape. We focus on the importance of DNA methylation to nucleosome stability, which may explain the context-dependent associations of DNA methylation with gene expression. Ultimately, we suggest that the integration of diverse epigenetic modifications in studies of insects will greatly advance our understanding of the evolution of epigenetic systems and epigenetic contributions to developmental regulation.

Introduction

Epigenetic information influences organismal phenotypes by making contributions to gene regulation that are transmissible through cell division (Bonasio et al. 2010). The two most widely studied forms of epigenetic marks are the methylation of DNA (Jaenisch and Bird 2003; Klose and Bird 2006; Zemach et al. 2010) and the posttranslational modification of histone proteins (Kharchenko et al. 2011; Suganuma and Workman 2011), both of which have been functionally implicated in the regulation of gene expression in a variety of taxa.

Epigenetic marks can be influenced by environmental variation and are capable of influencing postembryonic development (Jaenisch and Bird 2003). Such a role for intragenic DNA methylation was recently highlighted in the honey bee by Kucharski et al. (2008), who documented a developmental shift from the phenotype of workers to the phenotype of queens following the knockdown of DNMT3, an enzyme essential to *de novo* DNA methylation (Klose and Bird 2006). In light of this study, much attention has been devoted to the study of DNA methylation in insects (Elango et al. 2009; Foret et al. 2009; Hunt et al. 2010; Lyko et al. 2010; Xiang et al. 2010; Zeng and Yi 2010; Glastad et al. 2011; Park et al. 2011; Bonasio et al. 2012; Flores et al. 2012; Foret et al. 2012; Herb et al. 2012; Lockett et al. 2012; Patalano et al. 2012; Smith et al. 2012; Weiner and Toth 2012; Glastad et al. 2013; Snell-Rood et al. 2013). These studies have provided fundamental insights into the targets of DNA methylation in insect genomes, as well as the extent of differences in DNA methylation arising among distinct insect morphs. A lingering question remains, however: if DNA methylation plays a fundamental role in developmental regulation, why has it been lost in several lineages of insects? (Urieli-Shoval et al. 1982; Zemach et al. 2010; Glastad et al. 2011; Yi 2012).

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Table 1 Glossary of epigenomic terminology

Term	Definition
Chromatin	The packaged form of the eukaryotic genome. The fundamental unit of chromatin is the nucleosome, but chromatin also encompasses other DNA-binding proteins and protein complexes that bind nucleosomes.
DNA methylation	The addition of a methyl group to DNA. In animals, DNA methylation primarily affects cytosines occurring in a CpG context (cytosine followed by guanine in 5′–3′ orientation).
Epigenome	A broad term used to describe contributors to genome structure and function that are transmissible through cellular division, other than the DNA sequence itself.
Histone modification	One of many posttranslational alterations to a histone residue (e.g., methylation and acetylation). Distinct histone modifications exhibit distinct associations with genomic elements and gene regulation.
Histone variant	A histone encoded by a non-canonical histone gene. Many histone variants are incorporated into the nucleosome in a manner independent of replication, unlike canonical histones, and thus can replace canonical histones throughout the cell cycle.
Nucleosome	The fundamental, repeating unit of chromatin, comprised by ~147 base pairs of DNA wrapped around a histone octamer, each normally composed of two copies of the histone proteins H2A, H2B, H3, and H4.
Nucleosome positioning	The patterning of nucleosome occupancy along DNA and the extent to which this patterning is consistent among cells.
Nucleosome turnover	The eviction and replacement of the nucleosomes at a given position over time. A high degree of nucleosome turnover is associated with accessibility of DNA to protein binding and the incorporation of specific histone variants.

In this review, we highlight recent insights into the localization of DNA methylation in insect genomes and synthesize these results with insights into the eukaryotic epigenome made in model organisms (for a glossary of epigenetic terminology, see Table 1). We highlight the complementarity of the eukaryotic epigenetic landscape, which may be relevant to understanding the evolutionary loss of DNA methylation. We also discuss the importance of DNA methylation to nucleosome dynamics, which may explain the regulatory correlates of DNA methylation.

Selective localization of DNA methylation in insects

The localization of DNA methylation varies substantially among taxa. For example, vertebrate genomes are globally methylated (Suzuki and Bird 2008). In contrast, the genomes of invertebrates exhibit relatively sparse levels of DNA methylation, the majority of which is present within genes (Suzuki and Bird 2008; Feng et al. 2010; Zemach et al. 2010). Recently, the sequencing of single-base resolution profiles of DNA methylation on a genomic scale (DNA methylomes) has provided fundamental insights into the localization of DNA methylation in several insect taxa (Table 2).

Investigations of insect methylomes have demonstrated that, although the levels of DNA methylation in insect species vary substantially (Zemach et al. 2010; Sarda et al. 2012; Glastad et al. 2013; Hunt et al. 2013), the patterns of DNA methylation in insects with functional DNA methylation systems exhibit remarkable conservation (Fig. 1) (Zemach et al. 2010). In insects investigated to date, DNA methylation is highly biased to exons (Fig. 1b), highlighting the potential role of DNA methylation in exon definition and splicing (Feng et al. 2010; Lyko et al. 2010; Bonasio et al. 2012). DNA methylation in insect genomes is also primarily localized to the 5′-region following the translation start site of genes (Fig. 1a) (Zemach et al. 2010; Bonasio et al. 2012). In contrast to insects, in a basal chordate, *Ciona intestinalis*, intragenic DNA methylation is present throughout the length of gene bodies (Fig. 1a) (Zemach et al. 2010). This suggests that the conservation of DNA methylation in the 5′-region of genes may be particularly important in insects. It is noteworthy, however, that the localization of DNA methylation is unknown for many insect orders, including those most basal to the diversification of Insecta (Glastad et al. 2011). Thus, a full understanding of the targets of DNA methylation in insects requires further investigation of DNA methylation in a greater diversity of insect taxa.

In invertebrates, DNA methylation largely targets phylogenetically conserved genes (Hunt et al. 2010; Lyko et al. 2010; Sarda et al. 2012) that are constitutively expressed (Foret et al. 2009; Hunt et al. 2010). It is possible, however, that the inferred constitutive expression and phylogenetic conservation of methylated genes has been influenced by the use of whole bodies for assessing DNA methylation in insects (Table 2), and that data on DNA methylation

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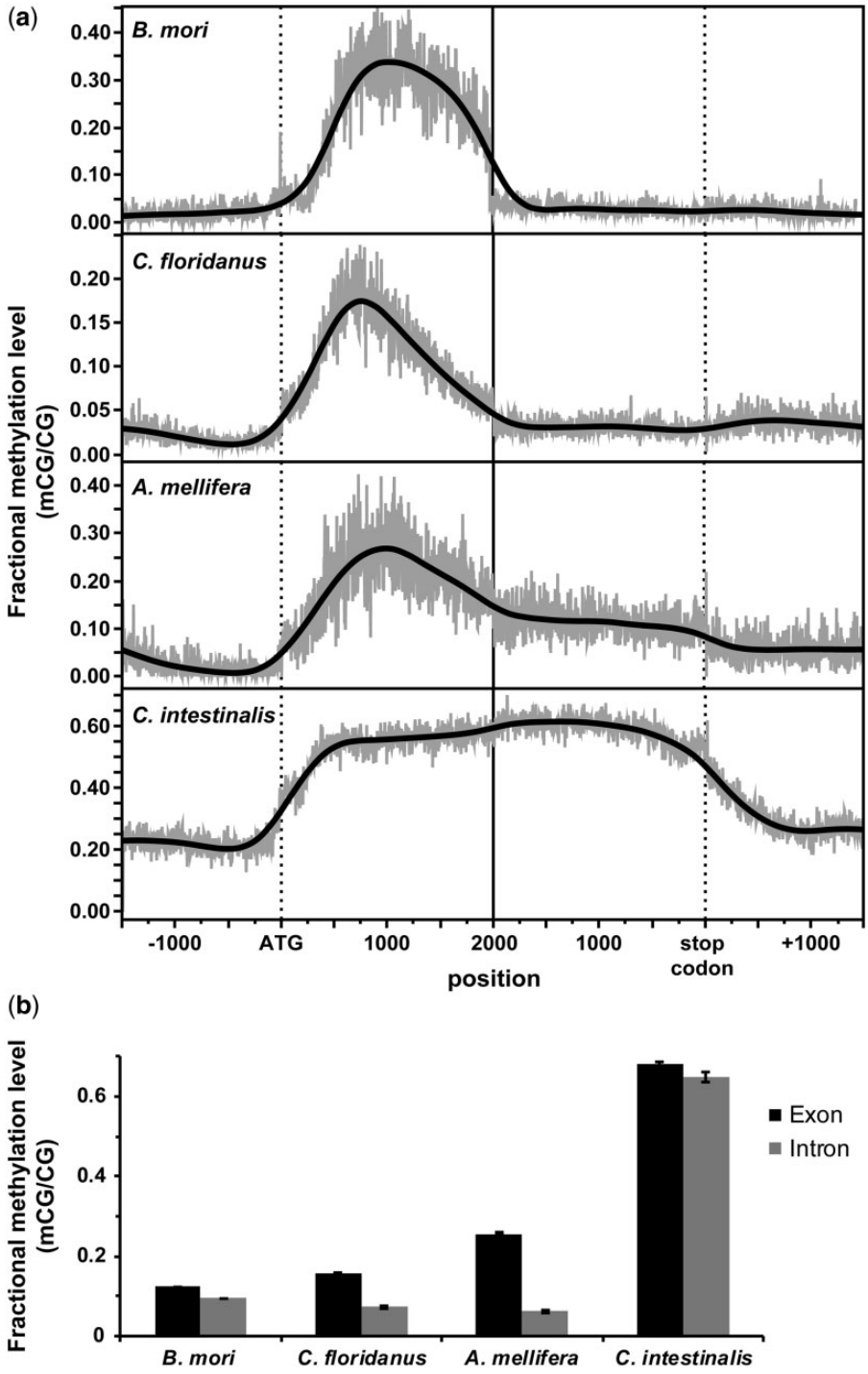

Fig. 1 Selective localization of DNA methylation in insect genomes. (**a**) The spatial profile of DNA methylation in genes greater than 4 kb in length in the silk moth *Bombyx mori* (Zemach et al. 2010), the carpenter ant *Camponotus floridanus* (Bonasio et al. 2012), and the honey bee *Apis mellifera* (Lyko et al. 2010; Zemach et al. 2010) exhibit preferential targeting of DNA methylation to the 5′-region of genes, immediately downstream of the translation start site (ATG). In contrast, DNA methylation in the genome of the invertebrate chordate *Ciona intestinalis* (Zemach et al. 2010), which diverged from arthropods roughly 900 million years ago (Hedges et al. 2006), is targeted throughout the length of gene bodies. The grey line connects mean fractional CpG DNA methylation values at each position and a smoother curve is shown in black. (**b**) DNA methylation is also preferentially targeted to exons (versus introns) in the insect taxa investigated; means with 95% confidence intervals are shown.

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Table 2 DNA methylomes from insect taxa

Species	DNA methyltransferases	Tissue
Apis mellifera (honey bee)	DNMT1, DNMT2, DNMT3	Whole body (Feng et al. 2010; Zemach et al. 2010), brain (Lyko et al. 2010), head (Foret et al. 2012)
Camponotus floridanus (carpenter ant)	DNMT1, DNMT2, DNMT3	Whole body (Bonasio et al. 2012)
Harpegnathos saltator (jumping ant)	DNMT1, DNMT2, DNMT3	Whole body (Bonasio et al. 2012)
Solenopsis invicta (red imported fire ant)	DNMT1, DNMT2, DNMT3	Whole body (Hunt et al. 2013)
Bombyx mori (silk moth)	DNMT1, DNMT2	Whole body (Zemach et al. 2010), silk gland (Xiang et al. 2010)

from individual cell types will reveal the presence of DNA methylation in tissue-specific genes. Regardless, insects with available methylomes also possess fewer methylated genes than do the highly diverged nonarthropod invertebrates, *Nematostella vectensis* and *C. intestinalis* (Sarda et al. 2012). Moreover, methylated genes in insects represent a subset of methylated orthologs identified in *N. vectensis* and *C. intestinalis* (Sarda et al. 2012). Likewise, DNA methylation targets largely overlapping sets of orthologs in the silk moth, the honey bee, and the fire ant (Sarda et al. 2012; Hunt et al. 2013), indicating that similar genes are methylated in distinct insect taxa. However, the difference in DNA methylation levels (Fig. 1b) and the number of genes targeted in insects, as compared with *N. vectensis* and *C. intestinalis*, suggest that a dramatic reduction in DNA methylation may have occurred in the arthropod lineage (Sarda et al. 2012).

Although great progress has been made in identifying the targets of DNA methylation, little is known about the greater epigenetic context of DNA methylation in insects. In the following sections, we review insights into the epigenome of eukaryotes. By synthesizing information on highly conserved components of the eukaryotic epigenome with the observed localization of DNA methylation in insect genomes, we hope to advance an understanding of the function of intragenic DNA methylation.

The epigenomic context of DNA methylation in insects

Studies of model mammalian and plant systems have provided great insight into understanding the complexity and interactive nature of the eukaryotic epigenome. For example, DNA methylation interacts with histone modifications and is associated with nucleosomes (Cedar and Bergman 2009; Chodavarapu et al. 2010). The regulatory roles of histone modifications are known to include mediation of the binding affinities of protein complexes, such as those related to transcriptional and splicing machinery (Luco et al. 2010; Bell et al. 2011; Luco et al. 2011; Negre et al. 2011; Bintu et al. 2012), as well as the direct alteration of local chromatin structure (Henikoff 2008; Bell et al. 2011; Bintu et al. 2012). Furthermore, several important histone modifications and structural variants show spatially heterogeneous patterns of enrichment within the bodies of actively expressed genes and are themselves predictive of gene activity (Roy et al. 2010; Ha et al. 2011; Kharchenko et al. 2011; Bieberstein et al. 2012; Bintu et al. 2012; Coleman-Derr and Zilberman 2012). Indeed, numerous empirical studies have demonstrated that histone modifications and variants influence transcriptional regulation (Henikoff 2008; Luco et al. 2010; Bintu et al. 2012).

Great strides have been made in profiling the chromatin landscape of the model insect, *Drosophila melanogaster* (Celniker et al. 2009; Filion et al. 2010; Roy et al. 2010; Kharchenko et al. 2011; Negre et al. 2011). For example, it has been shown that transcriptionally active, broadly expressed, genes are associated with multiple, specific histone modifications (Filion et al. 2010; Roy et al. 2010; Kharchenko et al. 2011). Similarly, DNA methylation is known to target actively transcribed genes in insects (Fig. 2a) (Foret et al. 2009; Zemach et al. 2010; Nanty et al. 2011). Moreover, the genomic localization of many histone modifications is highly conserved in diverse eukaryotic taxa (Bernstein et al. 2005; Feng and Jacobsen 2011; Woo and Li 2012; Simola et al. 2013). Comparative analyses of DNA methylation in non-model insects and histone modifications in *D. melanogaster* further suggest that DNA methylation is highly colocalized with several active histone modifications (Fig. 2) (Nanty et al. 2011; Hunt et al. 2013)

The function of DNA methylation in insects

The presence of DNA methylation in several insect lineages (Glastad et al. 2011), despite the highly

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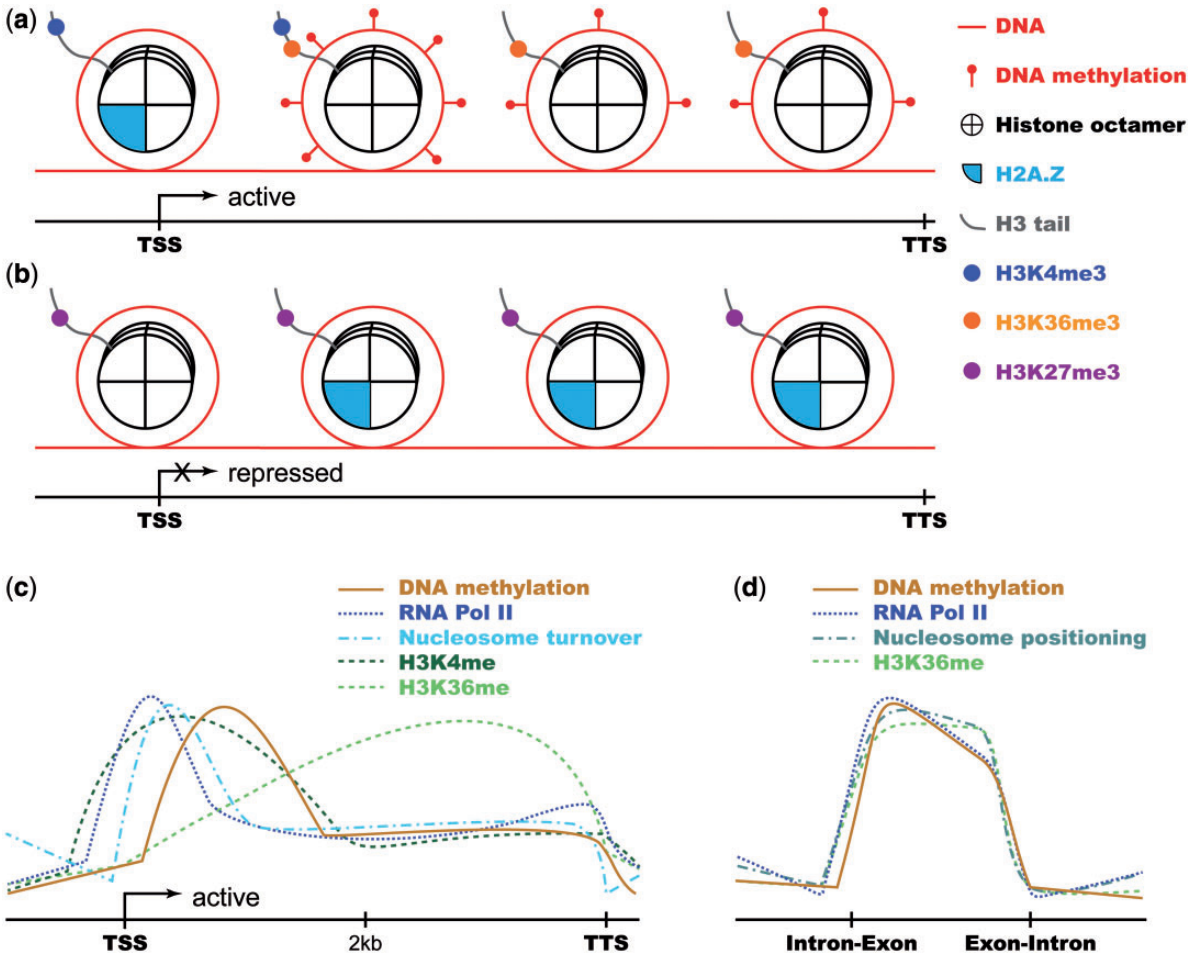

Fig. 2 A model of insect DNA methylation and the greater eukaryotic epigenome. (**a**) Actively expressed and (**b**) repressed (nonconstitutively expressed) genes exhibit distinct epigenetic profiles (Zilberman et al. 2008; Filion et al. 2010; Roy et al. 2010; Zemach et al. 2010; Bell et al. 2011; Zhou et al. 2011; Coleman-Derr and Zilberman 2012). Note that the histone modification H3K27me3 is largely limited to genes in regions of Polycomb-mediated repression, rather than in all nonconstitutive genes (Filion et al. 2010; Kharchenko et al. 2011). Model spatial profiles of enrichment signals for multiple epigenetic marks as visualized over (**c**) actively expressed genes (Mito et al. 2005; Deal et al. 2010; Roy et al. 2010; Zemach et al. 2010; Bell et al. 2011; Kharchenko et al. 2011; Yin et al. 2011; Adelman and Lis 2012; Bonasio et al. 2012) and (**d**) exons (Kolasinska-Zwierz et al. 2009; Schwartz et al. 2009; Chodavarapu et al. 2010; Kharchenko et al. 2011; Yin et al. 2011; Bonasio et al. 2012). (**a**, **c**) The spatial profile of DNA methylation targeting in constitutively expressed insect genes suggests DNA methylation may play a role in partitioning promoter regions and gene bodies. TSS, transcription start site; TTS, transcription termination site. Note that these generalized representations of spatial profiles are not meant to convey quantitative relationships between marks.

mutagenic nature of DNA methylation in animals (Elango et al. 2008), suggests that the methylation of DNA makes important functional contributions to insect epigenomes. It has long been hypothesized that one of the most important functions of DNA methylation is to influence the interactions between histones and DNA (Kass et al. 1997; Pennings et al. 2005). More recently, it has been demonstrated that DNA methylation alters the nucleosome by increasing the rigidity of histone-bound DNA, resulting in tighter wrapping of DNA around histones and a reduction in the linker DNA length between histone octamers (Choy et al. 2010; Lee and Lee 2011).

The fact that DNA methylation can alter the stability of the nucleosome has several important implications for studies of epigenetic gene regulation. For example, at high densities, RNA polymerase (Pol) II's traversal of the nucleosome can result in eviction of the nucleosome (Kulaeva et al. 2010), thereby exposing intragenic DNA-binding sites that would otherwise be occluded (Henikoff 2008). In this manner, eviction of nucleosomes can facilitate spurious initiation of intragenic transcription (Carrozza et al. 2005; Lieb and Clarke 2005). Should intragenic DNA methylation alter the potential for eviction of nucleosomes through its effects

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on nucleosome stability, then DNA methylation may play a role in suppressing recognition of spurious intragenic DNA-binding sites (Zilberman et al. 2007; Maunakea et al. 2010; Jones 2012). In support of this view, it is notable that genic regions exhibiting strong enrichment of RNA polymerase II coincide with regions of high density of DNA methylation (Fig. 2c).

DNA methylation has been linked to the positioning of nucleosomes (Chodavarapu et al. 2010), which has in turn been implicated in modulating intragenic RNA Pol II kinetics (Újvári et al. 2008; Schwartz et al. 2009; Luco et al. 2011; Luco and Misteli 2011; Bintu et al. 2012) that affect patterns of gene splicing (de la Mata et al. 2003; Kornblihtt 2005; Luco et al. 2011). Presumably, the positioning of nucleosomes is associated with definition of exons in invertebrates and vertebrates alike (Andersson et al. 2009; Schwartz et al. 2009; Tilgner et al. 2009). Thus, by influencing nucleosome stability and positioning, DNA methylation may contribute to the definition of exons during the transcription and splicing of mRNA.

These structural implications of DNA methylation may help to explain its context-dependent effects (Jones 1999, 2012). DNA methylation contributes to silencing of genes when present in promoters (Kass et al. 1997; Bird 2002; Pai et al. 2011; Zeng et al. 2012), but is associated with active expression when present within genes (exons + introns) (Zemach et al. 2010; Jjingo et al. 2012). These divergent relationships of DNA methylation with gene expression could be driven by a common role of DNA methylation in the stabilization of nucleosomes and in the occlusion of the initiation of transcription. Likewise, the introduction of a methyl group to DNA can directly alter the binding of transcription factors (Bird 2002; Shukla et al. 2011; Wang et al. 2012), further buffering against recognition of intragenic DNA-binding sites in actively expressed genes.

As discussed earlier, DNA methylation is primarily localized to the 5′-region of genes in the insect genomes studied to date (Fig. 1a) (Zemach et al. 2010; Bonasio et al. 2012). This 5′-region of the gene also exhibits enrichment of specific histone modifications (Roy et al. 2010; Henikoff and Shilatifard 2011; Kharchenko et al. 2011), elevated RNA Pol II density (Mito et al. 2005; Yin et al. 2011), and high rates of nucleosome turnover (Mito et al. 2005; Deal et al. 2010), relative to downstream regions of the gene (Fig. 2c). Together, these features may help to assign molecular context and functional attributes to DNA methylation in insect genomes, as described later.

Nucleosome turnover is elevated in actively expressed genes and is associated with the incorporation of specific variants of histones (Mito et al. 2005; Henikoff 2008; Deal et al. 2010; Bell et al. 2011). Moreover, the turnover of nucleosomes may be linked to increased accessibility to DNA-binding proteins (Henikoff 2008). Intriguingly, DNA methylation is highest slightly downstream of peak turnover of nucleosomes and maximum density of RNA Pol II in *Drosophila* (Deal et al. 2010; Roy et al. 2010; Zemach et al. 2010) (Fig. 2c). This spatial concordance highlights the possibility that DNA methylation is important to partitioning gene bodies and transcription start sites. Consistent with this hypothesis, DNA methylation exhibits a spatially antagonistic relationship with the histone variant H2A.Z in diverse eukaryotes (Zilberman et al. 2008; Conerly et al. 2010; Zemach et al. 2010; Coleman-Derr and Zilberman 2012). H2A.Z enhances recruitment of RNA Pol II at transcription start sites (Hardy et al. 2009), particularly in constitutively expressed genes (Coleman-Derr and Zilberman 2012). It is feasible that DNA methylation decreases intragenic transcription initiation by increasing nucleosome stability and by inhibiting the incorporation of H2A.Z (Talbert and Henikoff 2010; Coleman-Derr and Zilberman 2012) in 5′-regions of constitutively expressed insect genes (Figs. 1 and 2).

The close spatial association of DNA methylation and RNA Pol II in insect genomes (Figs. 2c and d) provides insight into the link between DNA methylation and the regulation of alternative splicing. Several studies in diverse eukaryotes have demonstrated that experimental alteration of RNA Pol II's processivity has direct effects on the outcome of alternative splicing (de la Mata et al. 2003; Kornblihtt 2005; Luco et al. 2011). This is presumably due to the molecular splicing machinery's variable recognition of splice sites, which depends upon both the speed of elongation of RNA Pol II and the strength of the signal of the splice site (Luco and Misteli 2011). DNA methylation may have a direct negative effect on the speed and elongation efficiency of RNA Pol II (Lorincz et al. 2004; Zilberman et al. 2007). Moreover, DNA methylation has been shown to mediate the binding of proteins, including the transcription factor CTCF (Wang et al. 2012). In humans, the mediation of CTCF binding by DNA methylation has been shown to affect the dynamics of RNA Pol II and alternative splicing (Shukla et al. 2011). These insights may be particularly relevant to transcriptional regulation in insects, given that DNA methylation levels are highest in exons in insect genomes (Feng et al. 2010; Lyko et al. 2010; Bonasio et al.

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2012) (Figs. 1b and 2d), and that an association has been observed between DNA methylation and alternative splicing in insects (Lyko et al. 2010; Park et al. 2011; Bonasio et al. 2012; Flores et al. 2012; Foret et al. 2012; Lockett et al. 2012).

Nevertheless, it is critical to note that DNA methylation is not the sole epigenetic contributor to the mediation of alternative splicing (Luco et al. 2010, 2011; Luco and Misteli 2011). The ''chromatin–adaptor complex'' model of the regulation of alternative splicing describes a mechanism whereby several histone modification-binding proteins interact with splicing factors to modulate the outcome of alternative splicing (Luco et al. 2011). Notably, several specific histone modifications are positively correlated with the presence of DNA methylation in insects (Hunt et al. 2013), suggesting that it may be particularly challenging to disentangle the relative contributions of histone modifications and DNA methylation to patterns of alternative splicing.

Conclusions

DNA methylation and histone modifications exhibit complementary, and potentially redundant, functions, which may help explain the loss of DNA methylation in some insect lineages (Zemach et al. 2010; Glastad et al. 2011). Nevertheless, the aforementioned links between DNA methylation and the dynamics of nucleosomes suggest that DNA methylation makes important functional contributions to the epigenome.

Many regulatory properties previously ascribed to DNA methylation are potentially driven, at least in part, by the association of DNA methylation with conserved epigenetic and regulatory genomic domains in insects, highlighting the role of DNA methylation as a single component of a complex epigenomic whole. This view leads to two fundamental unanswered questions about the regulatory roles of intragenic DNA methylation in insects: (1) How does the presence and absence of DNA methylation affect chromatin structure and gene regulation? and (2) Why are 5′-regions of insect gene bodies preferentially targeted by DNA methylation?

Insights into the regulatory significance of DNA methylation, and its loss, stand to be gained on two fronts. First, the loss of DNA methylation in some insect lineages provides the opportunity to take a comparative genomics approach to explore differences in chromatin structure and gene regulation between taxa with and without functional DNA methylation systems. Second, the experimental knockdown of DNA methyltransferase expression (e.g., Kucharski et al. 2008) can be used in conjunction with a comprehensive profiling of various epigenomic modifications (including histone variants, modifications, and nucleosome positioning) in order to explore the consequences of depletion of DNA methylation on chromatin structure and gene regulation.

In this review, we have commented on the functional significance of 5′-proximal localization of DNA methylation in the genes of insects. In particular, we have highlighted the proximity of DNA methylation to the localization of histone modifications associated with active transcription, regions of high nucleosome positioning, and presence of RNA Pol II. Furthermore, we suggest that DNA methylation may play a role in partitioning gene bodies and promoter regions of actively expressed genes by limiting accessibility to transcription factors in the 5′-region of gene bodies.

Studies that take a comprehensive approach to the insect epigenome, by profiling diverse components of the epigenome, will provide further insight into the importance of 5′-proximal intragenic DNA methylation. We believe the restricted localization of intragenic methylation in insects, coupled with the observed variation in DNA methylation levels among insect taxa (Fig. 1), make insects ideally suited to provide fundamental insights into the functional significance of intragenic DNA methylation.

Methods

Illumina reads from bisulfite-converted genomic DNA from *Apis mellifera* (Lyko et al. 2010; Zemach et al. 2010), *Camponotus floridanus* (Bonasio et al. 2012), *Bombyx mori* (Zemach et al. 2010), and *C. intestinalis* (Zemach et al. 2010) were mapped to reference genomes using Bismark (Krueger and Andrews 2011). Information on accession of data can be found in source publications. Fractional methylation values were calculated for each CpG site as mCG/CG, where mCG is the number of reads with a methylated cytosine at a CpG site (according to non-conversion) and CG is the total number of reads mapped to the site. Fractional methylation was calculated for specific genetic elements as the mean of all values of CpG fractional methylation within that element. Significantly methylated CpG sites were assessed using a binomial test, which provided a significance value to each CpG site. Resulting *P*-values were then adjusted for multiple testing (Benjamini and Hochberg 1995). Only CpG sites with false discovery rate (FDR) corrected binomial *P*-values <0.01, and ≥ 3 reads, were considered ''methylated.'' Figure 1 includes only genes 4 kb or

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longer in length, with ≥3 CpG sites called as methylated (after FDR correction), in order to reflect only those confidently targeted by DNA methylation.

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