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REVIEW ARTICLE

Epigenetics with special reference to the human X chromosome inactivation and the enigma of *Drosophila* DNA methylation

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Abstract. Epigenetics confers adaptability and survival advantage to an organism. Most epigenetic processes demonstrate memory and heritability. DNA methylation is an epigenetic process that adds imprints which can be inherited during cell division and across generations. DNA methylation adds an additional level of information to the basic DNA sequence and can influence chromatin organization and the function of the DNA sequence. In bacteria, it works as a defence strategy and preserves genome integrity. DNA methylation in eukaryotes has been implicated in a large number of cellular regulatory processes and is implied in development, differentiation, life style diseases and cancer. Mammals have an intricate DNA methylation machinery with dNMT1, 3A and 3B enzymes. The human X chromosome inactivation, an example of differential regulation of homologous chromosomes, is known to involve many epigenetic processes with intricate interactions of lnc RNAs, miRNAs and DNA methylation. *Drosophila* possesses very low levels of DNA methylation with only *dNMT2* gene. Since *Drosophila* is an important model organism for study of development and differentiation, the implications of this sparse DNA methylation and the lack of DNA methylation machinery in *Drosophila* is discussed.

Keywords. 5-methylcytosine and 6-methyladenine; DNA methyltransferases; chromatin remodelling; CpG methylation.

Epigenetic processes involve chromatin remodelling and imprinting. Epigenetic marks are set in place and maintained through DNA methylation, modifications of proteins such as histones and remodelling partners, transcription activating factors and miRNAs (Kim *et al.* 2009). Epigenetics is important in normal development and differentiation, adaptation to stress, ageing, cancer and life style related diseases (Laird and Jaenisch 1996; Jones and Baylin 2007). Epigenetics has been implied in the cross talks between myriads of processes with crucial regulatory roles (Allis and Jenuwein 2016).

DNA methylation is a postreplicative modification of DNA carried out by a DNA methyltransferase using SAM as a methyl donor. It can alter DNA protein interactions, DNA conformation and chromatin structure thereby influencing activity and functional states. It involves distinct DNA methyltransferases namely, DNMT1, DNMT2, DNMT3A and DNMT3B (figure 1) (Jurkowska and Jeltsch 2016). DNA methylation patterns vary across development and differentiation in cells and tissues (Barlow and Bartolomei 2014). Studies with identical monozygotic twins and altered nutritional and physiological states have demonstrated that these are characterized by specific but diverse patterns of DNA methylation (Cooney *et al.* 2002; Fraga *et al.* 2005). DNA methylation has been implicated in several functions during development, throughout the life processes and in ageing. Although genetic changes are thought to be the primary drivers of many cancers, in more than 50% cancers, DNA methylation patterns can be correlated with origin, aggressiveness and progression of cancer (Jones and Baylin 2007).

Microbes

A primary function of DNA methylation in microbes is preservation of the genetic material and genome integrity. In microorganisms, DNA cytosine and adenine methylation are involved in the restriction modification systems (Sánchez-Romero *et al.* 2015) which are important in preserving genetic identity of an organism. It works to prevent incoming foreign DNA from getting internalized in the bacteria. DNA adenine methylation plays a role

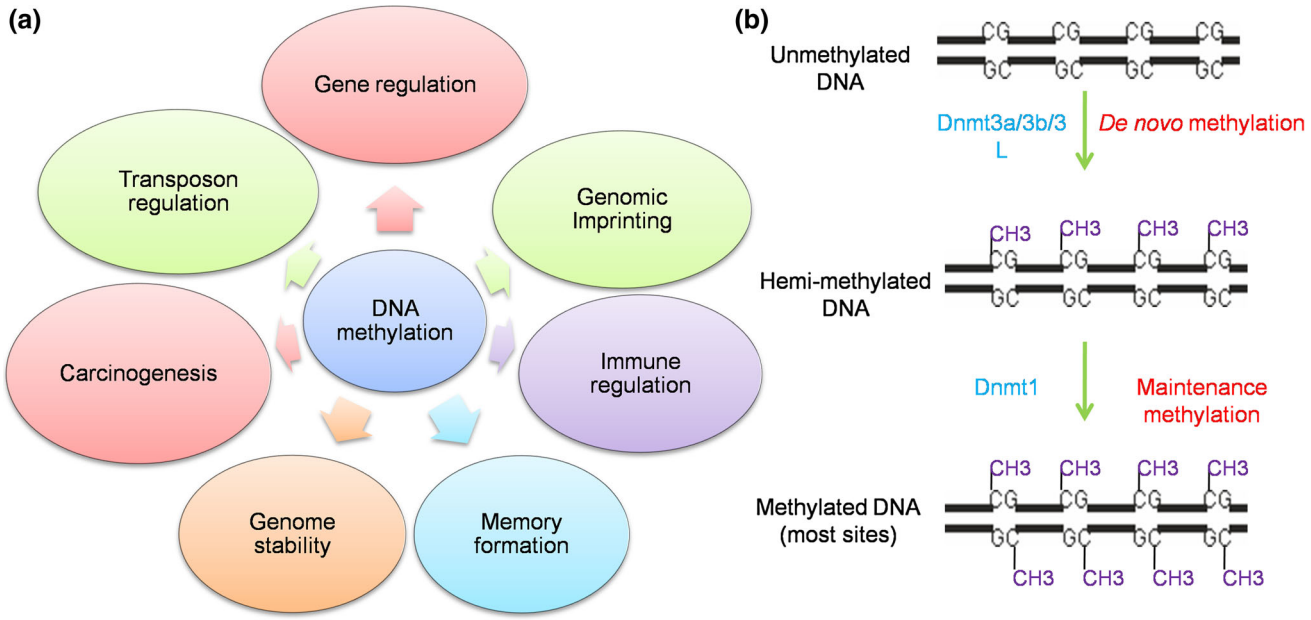

Figure 1. (a) Summary of functions of DNA methylation in various organisms. DNA methylation plays a role in various processes throughout the development in plants, invertebrates and vertebrates. (b) *De novo* and maintenance methylation.

in conferring strand discrimination in mismatch repair (Radman and Wagner 1986). This elegant process ensures that the mismatches in the newly replicated strand of the DNA are selectively repaired. DNA methylation influences gene expression (Low and Casadesús 2008) and is known to regulate virulence and phase variation (Marinus and Casadesus 2009). *Deinococcus radiodurans*, the world's most radiation and desiccation resistant microbe, harbours methylation machinery which comprises of an adenine methyltransferase which plays a role in metabolic rewiring (Prasad *et al.* 2005; Shaiwale *et al.* 2015; Patil *et al.* 2017) and an unusual cytosine DNA methyltransferase (Patil *et al.* 2017). When as many as 230 diverse bacterial and archaeal genomes were analysed, evidence of DNA methylation was found in 215 microbes (93% of those sequenced) and over 600 DNA enzymes (methyltransferases) were annotated (Blow *et al.* 2016). While several DNA methylation enzymes are part of the restriction modification systems (consistent with their known role in defence against viruses), a substantial number of them appeared to be involved in genome regulation, and have a more crucial and perhaps critical role in prokaryotic physiology and biology (Blow *et al.* 2016). The role in restriction modification systems are confined to microbes. As the organisms have evolved, the functions of DNA methylation have undergone changes, with methylation assuming more regulatory role in eukaryotes in epigenetic processes involved in chromatin structure and adaptation.

DNA methylation in mammals

In mammals, DNA methylation is involved in regulating gene expression, chromatin structure and imprinting (Deobagkar *et al.* 1990). Mutations in genes encoding DNA methyltransferases demonstrate embryonic lethality (Li *et al.* 1992). Several roles played by DNA methylation have been reviewed previously (Herman *et al.* 1995). In mammals, DNA methylation plays a crucial role in development (Li *et al.* 1992; Okano *et al.* 1999; Smith and Meissner 2013), cancer initiation and progression (Jones and Baylin 2007) and shows transgenerational transmission. DNA cytosine methylation pattern shows an alteration in response to nutrition, environmental cues (Jones and Baylin 2007) and upon exposure to stress and toxicants (Deobagkar *et al.* 2012). Recently, novel autoregulatory aspects of DNA methyltransferases have been revealed which involve N-terminal domain and post-translational modifications (Jetsch and Juroskava 2016). The mechanisms of mammalian X chromosome inactivation have been extensively studied and are reviewed in the following section as a 'case study' to elaborate the complex interplay in the epigenetic regulation.

X chromosome inactivation

X chromosome inactivation in female mammals is an example of differential regulation of homologous chromosomes. Mary Lyon (Rastan 2015) suggested the hypothesis of X chromosome inactivation to explain the presence of the Barr body in female mammals (Rastan 2015). In eutherian mammals and marsupials, one of the two X chromosomes undergoes inactivation. Imprinting and epigenetic processes have been implicated in X chromosome inactivation (Prothero *et al.* 2009; Migeon 2017). In marsupials, the paternal X is inactivated, and in mice,

in extraembryonic tissue, the paternal X chromosome is inactivated while in the embryo proper, the X inactivation is random. The inactive X chromosome (Xi) is late replicating, heterochromatic and transcriptionally silenced (Gartler and Riggs 1983). It has been well documented that in mammals, one X chromosome is marked as active while the other gets inactivated in all the cells. Mammals have evolved an elaborate mechanism for establishing and maintaining the inactive X chromosome. X chromosome inactivation has been suggested to be an important process in sex determination (Chandra 1985). For a particular X chromosome to undergo inactivation, presence of X chromosome inactivation centre (XIC) or X chromosome control element (XCE) is important. X inactivation specific transcript (XIST) and its antisense transcript, Tsix (Plath *et al.* 2002) are transcribed from XIC. XIST is transcribed exclusively from the inactive X chromosome, coats the inactive X chromosome and has been shown to be an important component of the X inactivation machinery. X activation specific transcript (XACT), a long noncoding RNA (lncRNA) is seen to localize to active X chromosome in human pluripotent stem cells (Vallot *et al.* 2013). Other partners, namely noncoding RNAs RepA, JpX, FtX and PcG polycomb group of proteins, are shown to interact with Xist in establishing the X chromosome inactivation (Zhao *et al.* 2008; Tian *et al.* 2010; Soma *et al.* 2014). In mouse blastocyst, Xist is regulated by transacting protein factors, namely Oct4, Sox2, Nanog and Rex1 (Silva *et al.* 2009; Gontan *et al.* 2012). Rnf12/RLIM (Jonkers *et al.* 2009) which encode the ubiquitinase enzyme are also involved in the silencing of the X chromosome. In addition to silencing, Xist RNA has multiple roles in XCI such as spatial reorganization of the X chromosome and chromatin remodelling. As a consequence of Xist coating, a subnuclear 'domain' is created thereby silencing genes during X chromosome inactivation (Chaumeil *et al.* 2006). The Xist lncRNA is involved in multiple steps during X inactivation, including coating of the presumptive inactive X (Xi) chromosome, exclusion of RNA polymerase, reorganization of chromatin into inactive domains, methylation of DNA, localization of the inactive X to the nuclear periphery and packing of the chromatin (Lu *et al.* 2017). Another X-linked lncRNA Firre is involved in regulation of chromatin conformation, (CTCF) binding and methylation of histone H3K27me3 and helps in positioning of inactive X chromosome in the nucleus (Yang *et al.* 2015). Methylation of histone H3 at Lys-9 has been recognized as an early chromatin mark on the inactive X chromosome (Heard *et al*. 2001). Differential expression of miRNAs has been reported in cells with varying number of inactive X chromosomes (Rajpathak and Deobagkar 2017a). In XO Turner cases, these differentially expressed miRNAs appear to be participating in the epigenetic processes and are involved in various pathophysiological conditions observed in a Turner patient. These include aneuploidy, obesity, cancer, type-I diabetes, renal senescence, neural development and differentiation (Rajpathak and Deobagkar 2017b). Five differentially expressed lncRNAs in human X monosomy cells have been reported (Rajpathak *et al.* 2014). Further analysis (using DIANA LncBase V2) led to the identification of four (miR-10b-5p, miR-125a-5p, miR-4325 and miR-615-5p) miRNAs which can interact with lncRNA Xist and are differentially expressed in 45, XO cells. It has been suggested that some lncRNAs can act as molecular 'sponges' of miRNAs and titrate away the active miRNA thereby influencing the expression of genes.

DNA cytosine methylation is involved in X chromosome inactivation and early reports have shown that treatment with 5 azaC, a known demethylating agent, could reactivate the inactive X chromosome (Mohandas *et al.* 1980). DNMT1 knockout mice embryos also showed reactivation of X-linked genes (Sado *et al.* 2000). SmcHD1 (Blewitt *et al.* 2008) and alpha thalassaemia / mental retardation X-linked protein (Baumann and De La Fuente 2009) have been implicated in the maintenance of X chromosome inactivation. By employing photo acoustic spectroscopy the amount of DNA cytosine methylation was shown to increase linearly with the number of inactive X chromosomes in the human fibroblast cells with varying number of X chromosomes (namely XO, XX and XXX chromosomes) (Achwal *et al.* 1984; Deobagkar and Chandra 2003). There are reports of methylation of CpG islands on the inactive X chromosome (Pfeifer *et al.* 1990; Hellman and Chess 2007). The inactive X chromosome has more methylation in the repeats, transposons and LINE elements along with genic regions (Patil *et al.* 2014). A comprehensive map of tissue-specific pattern of gene expression for the X-linked genes has been recently reported (Tukiainen *et al.* 2016).

X chromosome has evolved sequence patterns which facilitate setting up of inactivation by the remodelling machinery. Thus sequences that are responsible for the inactivated state to spread may be enriched along the X chromosome and could be present as 'way stations' on the X chromosome (Riggs 1990). Such sequences could potentially be docking sites for a molecule like Xist that coats the inactive X chromosome and could be responsible for the maintenance and spread of inactivation. There have been studies using X autosome translocations and transgenes inserted into the X chromosome that report partial silencing or even escape from inactivation of autosomal regions (Lee and Jaenisch 1997). Along the X chromosome, motifs from L1 or LINE1 are enriched in sequences that undergo inactivation whereas they are absent from regions that escape inactivation (Wang *et al.* 2006). We have designed a sensitive high throughput microarray-based immunochemical approach to detect methylation of a gene or a region (Kelkar and Deobagkar 2009; Kelkar and Deobagkar 2010; Deobagkar *et al.* 2012; Rajpathak and Deobagkar 2014; Rajpathak *et al.* 2014) and

examined the human diploid fibroblast cell lines with varying number of inactive X chromosomes, namely 45, X (no Xi), 46, XX (one Xi) and 47, XXX (two Xi). This has helped in generating the methylation map of the active and the inactive X chromosome in human (Rajpathak and Deobagkar 2014). X chromosome has been distributed into various strata based on the evolutionary origin of the sequences on the chromosome. When the locations of consistently methylated genes were examined most of them belonged to the S1, namely the earliest evolutionary strata, implicating that regions of X chromosome which were acquired early in evolution had more stable and consistent methylation pattern. It appears that sequences on the X chromosome have evolved differently from other chromosomes, so that the process of inactivation can identify these domains and thereby maintain stable inactive states (Kelkar and Deobagkar 2009; Kelkar *et al.* 2009). As many as 25% genes from the inactive X chromosome show partial or complete escape from inactivation and most of these genes localize on the recently acquired regions (Cotton *et al.* 2013; Cotton *et al.* 2014; Disteche and Berletch 2015). Aneuploidy (missing the second X or Y chromosome) led to the misregulation of the epigenetic machinery and this altered methylation (BMP2, IGF1) correlated well with phenotypes of the XO Turner syndrome (Rajpathak and Deobagkar 2017a,b). These included not only the genes involved in setting epigenetic marks but also genes encoding phenotypes related to bone remodelling, growth, thyroid metabolism, glucose and sugar metabolism and sex differentiation, e.g. ovarian development. Since misregulation of epigenetic machinery appears to be important in establishing phenotypes of Turner syndrome, epigenetic, in particular methylation modulators, can have therapeutic potential for treatment of phenotypes of Turner syndrome. Novel methylation inhibitors have been designed which will find applications in the treatment of cases XO Turner, cancers and many other lifestyle diseases (Gawade *et al.* 2016; Joshi *et al.* 2016). Mutations or modulation in the methyltransferase machinery or its regulation have also been implicated in many human diseases (Hamidi *et al.* 2015).

Previously it has been reported that DNMT1-depletion led to global DNA hypomethylation and this destabilized the cells leading to aneuploidy (Barra *et al.* 2012). Both DNA methylation and expression of DNA methyltransferase 1 increased with the increase in the number of inactive X chromosomes in cells with increasing number of inactive X chromosomes (namely aneuploid 45, XO, 46, XX and 47, XXX) (Rajpathak and Deobagkar 2017a,b).

There are several interesting features of the epigenetic machinery and its regulation in relation to the X chromosome inactivation with respect to the mechanism of silencing, chromosome architecture, imprinting which will remain the subject of further analysis (Bonora and Disteche 2017).

DNA methylation in insects with particular reference to *Drosophila*

Until recently, insect DNA methylation has not been a subject of extensive research. Honey bees have evolved an intricate caste system and possess DNA methylation along with the entire enzymatic machinery, namely the DNMT1, DNMT3A and DNMT3B. Although recently discovered, the role of DNA methylation is implied to be important in gene expression and social behaviour in this eusocial insect (Elango *et al.* 2009). DNA methylation is reported to be within the coding sequence and may have some role in the caste system consisting of workers and the queen (Foret *et al.* 2012). It has been suggested that DNA methylation in honey bees affects life span (Cardoso-Júnior *et al.* 2017).

Insects have therefore emerged as an interesting model to delineate novel roles for DNA methylation. In carpenter bees, which is a sub social insect, DNA methylation is targeted to its exons (Rehan *et al.* 2016). Queen pheromones have been shown to modulate DNA methyltransferase activity in bees and ant workers (Holman *et al.* 2016). It has been hypothesized that in Hymenoptera (bees, ants, wasps and sawflies), the evolution of eusocial division of labour is associated with DNA methylation in the genomes of Hymenoptera (bees, ants, wasps and sawflies). However, this has been questioned in a recent study (Glastad *et al.* 2017).

Drosophila is a model organism for genetic analysis of development and has been utilized extensively as a model system for human diseases, neural differentiation and evolving many basic concepts including the homeotic genes, developmental pattern formation, remodelling machinery. However, *Drosophila* is strikingly different with respect to DNA methylation machinery. It has been a topic of much debate from the 1980s with reports either claiming presence (Achwal *et al.* 1983; Achwal *et al.* 1984; Gowher *et al.* 2000; Lyko *et al.* 2000; Phalke *et al.* 2009) or absence of DNA cytosine methylation (Urieli-Shoval *et al.* 1982; Patel and Gopinathan 1987; Raddatz *et al.* 2013). DNA methylation in *Drosophila* has remained enigmatic for several years. *Drosophila* along with other Dipterans, lacks DNA methyltransferases DNMT1/3A/3B and possesses only DNMT2 (Tang *et al.* 2003). DNMT2 was identified as a DNA/RNA methyltransferase (https://www.brenda-enzymes.org), particularly with reference to lower eukaryotes. DNMT2 knockout flies have reduced life span, compromised immune function and sensitivity to stress (Durdevic *et al.* 2013). Recently, two independent LC-MS/MS based studies have reported the presence of 5mC in the genome of adult *D. melanogaster* (between 0.01 and 0.034% of cytosine) (Capuano *et al.* 2014; Rasmussen *et al.* 2016). Presence of DNA cytosine methylation was confirmed in specific DNA sequences in *Drosophila* genome by selective enrichment of methylated DNA followed by bisulphite sequencing in stage 5 embryos (Takayama *et al.* 2014). Rasmussen *et al.* (2016) quantified 5mC in adult

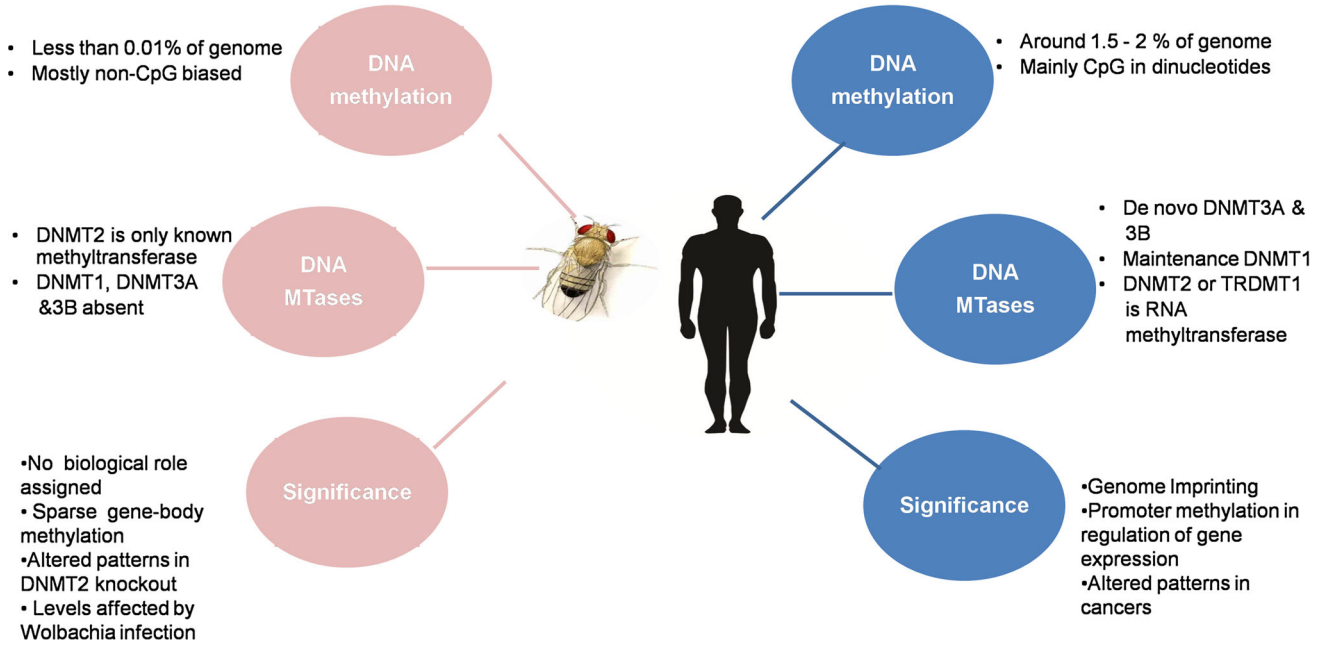

Figure 2. Comparison between *D. melanogaster* and mammalian methylation systems.

D. melanogaster and reported a lower 5mC level in fruit flies than in honey bees. Using methylation microarray-based technique; the changes in pattern of DNA methylation during embryonic, pupal and adult stages of *D. melanogaster* were identified (Panikar *et al.* 2017). Using a novel microarray approach, the presence of an active DNA methyltransferase was demonstrated in protein extract from S2 cells (Pannikar *et al.* 2004, 2015). Interestingly, presence of another epigenetic modification 6 methyladenine (6mA) was detected earlier in the genome of *D. melanogaster* (Achwal *et al.* 1983). Recently, by employing UHPLC-MRM-MS/MS technique, the presence of 6mA was reported in the embryonic stage of fruit flies (Zhang *et al.* 2015). This study also suggested that DNA 6mA demethylase, Drosophila TET homolog (DMAD), catalyses 6mA demethylation *in vitro*. Although 6mA has been reported to be present in *Drosophila*, there is no report on a putative DNA methyltransferase which can generate 6mA. In general, there are very few reports on presence of 6mA in eukaryotes (Heyn and Esteller 2015) and the enzymatic machinery remains to be identified or studied.

When the presence of methylation was analysed in the DNMT2 null mutants, 5mC was detected in the genomic DNA, albeit with an altered methylation pattern (Takayama *et al.* 2014). From this evidence, it appears that although dNMT2 may participate in the DNA methylation in *Drosophila* or may modulate the pattern, DNA methylation is present in the genomic DNA even in a DNMT2 null mutant. The search for a functional DNA methyltransferase in *Drosophila* genome and proteome needs to be carried out. It is interesting to note that *Drosophila* has been reported to have nonCpG methylation that is methylation in CpA or CpT dinucleotides (asymmetric) (Chatterjee *et al.* 2004; Takayama *et al.* 2014; Epigenetic regulation, stress and adaptation in Drosophila development, Deshmukh 2018, Ph.D. thesis). It will be interesting to unravel how this may be inherited across cell replication and development and differentiation. It can thus be concluded that although *Drosophila* has been employed as a model system for development, cancer, apoptosis etc., it shows distinct differences with respect to an important aspect of the epigenetic machinery (figure 2) and appears to manage very well without the DNMT1, 3A and 3B type of methylation. Our studies on methylation in *Drosophila* have led to the demonstration of changes in lipid metabolism along with a distinct suppression of immune function in both cellular and humoral arms associated with ageing in DNMT2 mutant flies (Epigenetic regulation of pathogenic stress and innate immunity genes, Abhyankar 2018, Ph.D. thesis). This could be due to the role of DNMT2 protein as an RNA methyltransferase or altered methylation in *Drosophila*. Patterns of methylation vary during development and life cycle stages (Epigenetic regulation, stress and adaptation in Drosophila development, Deshmukh 2018, Ph.D. thesis; Panikar *et al.* 2017). It will be very interesting to explore how the fruit fly compensates for the lack of methylation machinery. The DNA cytosine methylation present in *Drosophila* is sparse, asymmetric and has not been assigned any biological role. *Drosophila* appears to manage with the little methylation it possesses. Has *Drosophila* evolved alternate regulatory mechanisms to compensate for this loss? The search for the active DNA methyltransferase for both the adenine

and cytosine methylation in *Drosophila* continues and further analysis is likely to reveal novel features of the fine tuning of the epigenetic machinery.

Summary

DNA methylation thus has a pivotal role in epigenetic processes. DNA methylation seems to have evolved in multicellular organisms to further enrich the messages encoded within the DNA sequence to add newer connotations and meaning. Nutrition and environment orchestrate phenotypes by interplaying with the basic genetic information and allowing subtle changes. It is hence important to unravel the signals which decide the exact locations of methylation marks and imprints. The powerful model organism fruit fly and the human have major differences in the DNA methylation machinery. A basic understanding of the molecular genetic mechanisms in adding the epigenetic marks and interpreting their meaning will throw further light onto the networks governed by readers and writers of epigenetic processes and help design better strategies for treatment of cancer and life style diseases.

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