

DNA methylation changes in a gene-specific manner in different developmental stages of *Drosophila melanogaster*

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Although genomic DNA of *Drosophila melanogaster* has been shown to contain little cytosine methylation, the distribution of this genome-wide methylation patterns in different life stages remains to be elucidated. We have developed an immunochemical method using cDNA microarray to assess methylation. In the present work, this methylation microarray method was employed to identify DNA methylation in and around the genes in different life stages of *D. melanogaster*. This led to the identification of methylated genes in three stages of *D. melanogaster*, viz. embryo, pupa and adult. It is noteworthy that there was differential methylation in genes in different life cycle stages. Remarkably, a few functional annotation clusters showed negative correlation between transcription of a particular gene and its methylation status. In this analysis, some of the genes attributed to characteristic biological processes of particular life stage in *D. melanogaster* were found to be methylated in other life stages. Our analysis while providing a methylation map also suggests that gene-specific DNA methylation is altered during the life cycle stages of *D. melanogaster*.

Keywords: Developmental regulation, DNA methylation, *Drosophila* development, epigenetics, gene-specific methylation, 5 methyl cytosine.

DNA methylation is involved in the regulation of several molecular processes in an eukaryotic system. Methylation plays an important role in gene silencing^{1,2}, chromatin remodelling³ and repression of transposon activity⁴. In vertebrates, X chromosome inactivation and genomic imprinting are influenced by DNA methylation, wherein these phenomena are essential for the normal development of an organism. Invertebrates such as insects show significantly distinct life stages and developmental stage-specific regulation of gene expression which plays a crucial role in the transitions between differentiated life-history stages. Many research groups have emphasized

the presence of DNA methylation in invertebrates and have advocated that DNA methylation might be the mechanism for developmental gene regulation involved in life cycle transition⁵⁻¹⁰. The genome-wide DNA methylation maps of insects like *Drosophila melanogaster*⁹, *Bombyx mori*¹¹, *Solenopsis invicta*¹² and *Apis mellifera*^{13,14} have been generated using whole genome bisulphite sequencing. Therefore it is important to study whether these methylation signatures play an important role in life cycle processes in invertebrates.

Though *Drosophila* lacks the canonical DNMT1, DNMT3A and DNMT3B, the presence of very low levels of DNA methylation has been reported in *D. melanogaster*¹⁵⁻²². Presence of methylated DNA has been a controversial issue since few reports suggest that *D. melanogaster* lacks DNA methylation and DNMT2 is indeed an RNA methyltransferase that uses a DNA methyltransferase type of mechanism²³⁻²⁵. Capuano *et al.*¹⁹ employed a sensitive LC-MS/MS method to report the presence of 0.034% cytosine DNA methylation in *D. melanogaster*. DNA methylation in embryonic stage 5 of *D. melanogaster* was demonstrated by employing immuno-precipitation and bisulphite sequencing²⁰. These observations indicate that immuno-affinity based assays using anti-5-methyl cytosine antibodies have led to detection of DNA methylation in *D. melanogaster*^{15,20,26-30}.

Although 5'-cytosine methylation has been detected in different life stages of *D. melanogaster*^{15,16,20}, information on methylome map across the different life cycle stages of *D. melanogaster* is lacking. Assessment of genome wide DNA methylation in different developmental stages will provide insight into understanding the probable role of methylation during the development of *D. melanogaster*. In this context immunochemical techniques offer an advantage in enriching methylated DNA having low level of methylation^{18,27,28}. We had earlier employed an immunochemical method which exploits cDNA microarray to generate the methylome map in human and mouse³¹⁻³⁴. In the current study, methylation microarray provided us the means to generate the methylome of three distinct stages of *D. melanogaster*, viz. embryo, pupa and adult. Our results suggest that

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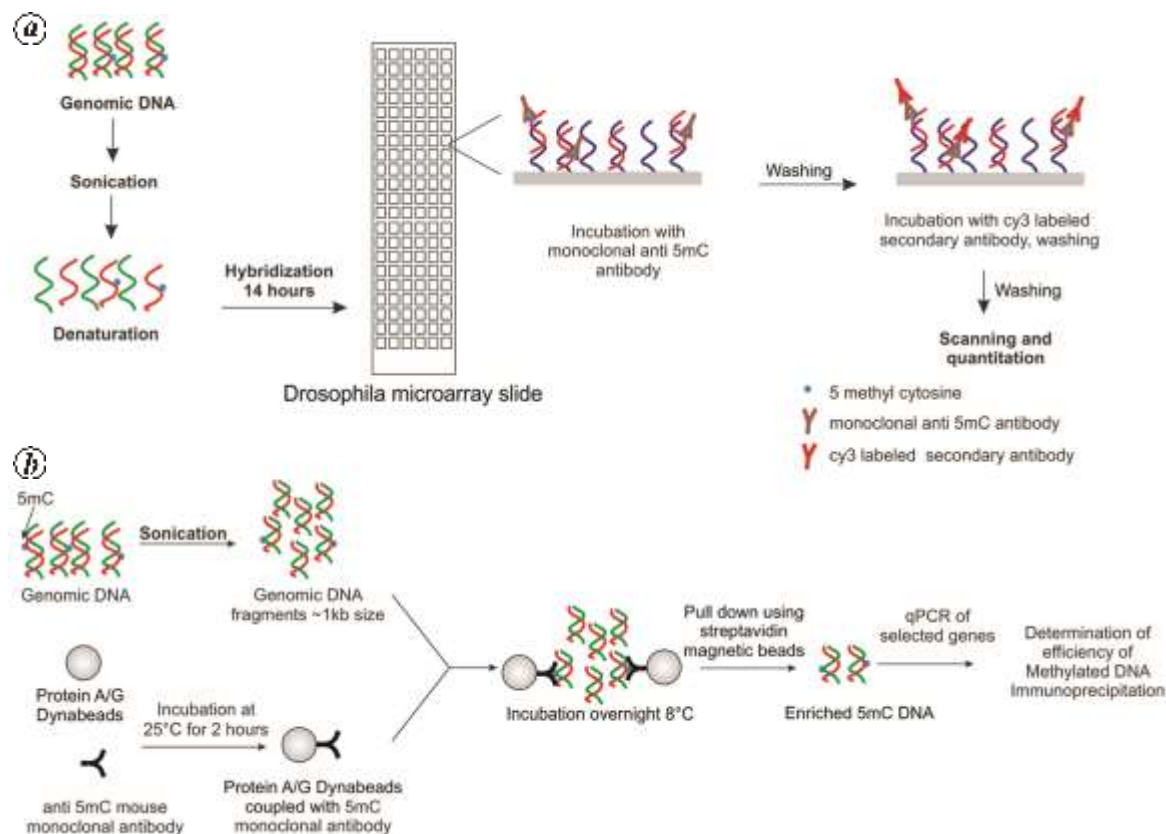


Figure 1. Schematic representation of (a) methylation microarray and (b) efficiency of methyl DNA immuno-precipitation.

DNA methylation might play an important role during the development of *D. melanogaster*.

Materials and methods

D. melanogaster culture

D. melanogaster ORK strain was cultured and maintained in laboratory conditions at 23°C. *D. melanogaster* adult (2–3-days-old), pupa (white pupa 8–10 h) and embryo (8–10 h) were used for the experiments. Three independent biological replicates were carried out.

Methylation microarray

Methylation microarray was carried out using methods described earlier³¹ (Figure 1a). Microarray slides were procured from the Toronto microarray centre (Toronto University, Canada) containing 28,000 *Drosophila* probes, representing 12,000 genes.

Antibodies

In-house developed anti-5-methyl cytosine mouse monoclonal antibodies were used in methylation microarray

and immuno-precipitation experiments³¹. Anti-mouse IgGs were labelled with cy3 using the cy3 antibody labelling kit (GE Healthcare life Sciences, USA). Genomic DNA was isolated as described earlier²⁷.

Methylated DNA immuno-precipitation (MeDIP)

Genomic DNA (5 µg) extracted from different developmental stages of *D. melanogaster* was subjected to sonication to generate random fragments ranging in size from 800 to 1000 bp. By employing protein A/G magnetic beads (Pierce, USA) and anti-5-methyl cytosine mouse monoclonal antibodies specific for methylated DNA, were used in immuno-precipitation pull-down experiments. Anti-5-methyl cytosine mouse monoclonal antibodies were immobilized on protein A/G beads as per instructions of manufacturer (Pierce, USA). DNA was first incubated with uncoupled beads to reduce the non-specific binding of DNA to beads. Anti-5-methyl cytosine mouse monoclonal antibody coupled with magnetic beads was incubated overnight with DNA (pre-cleared by protein A/G magnetic beads) at 8°C with continuous shaking. The DNA/antibody/beads complexes were washed twice with high stringency wash buffer (10 mM Tris-cl pH 7.5, 1 mM EDTA pH 8, 2 M NaCl), low stringency wash buffer (10 mM Tris-cl pH 7.5, 1 mM EDTA

pH 8, 150 mM NaCl) and finally with TE buffer (10 mM Tris-cl pH 8, 1 mM EDTA pH 8). Elution was done by re-suspending the beads in elution buffer (Pierce, USA). The no anti-5-methyl cytosine antibodies and no genomic DNA control IP reactions were performed. Schematic representation of MeDIP is shown in Figure 1 b.

qPCR analysis for immuno-precipitated DNA

Primers for selected genes were synthesized from IDT (USA) ([see Supplementary Information S1 online](#)). Real-time PCR on MeDIP samples was carried out using a Step-One-plus Real-Time PCR system (Applied Biosystems, USA). Universal thermal cycling conditions were used for detection of candidate genes: 50°C for 2 min, then 95°C for 10 min, followed by 95°C for 15 sec, and 60°C for 1 min, repeated for 40 cycles. RpS7, GAPDH and Rpn3 were used as negative control. Schematic representation of MeDIP qPCR is shown in Figure 1 b.

The efficiency of methylated DNA immuno-precipitation of particular genome loci was calculated from qPCR data and reported as a % methylated DNA-IP/total input DNA

$$\% \frac{\text{MeDNAIP}}{\text{total_input}} = 2^{[(Cr(10\% \text{input}) - 3.321) - Cr(\text{MeDNAIP})]} \times 100\%.$$

Gene ontology

Pathway and functional analysis of the genes was performed using database for annotation, visualization and integrated discovery (DAVID) Gene Ontology tool (<http://david.abcc.ncifcrf.gov/>)^{35,36} and PANTHER database available online (<http://www.pantherdb.org/>)³⁷.

Gene expression data retrieval

Mapped RNA-sequence reads from 10 to 12 h embryos, white pupa 12 h and adult 5 days were downloaded from the modMine database (<http://intermine.modencode.org/>)³⁸.

Detection of 5-methyl cytosine in embryo

Embryos were dechlorinated using 5% hypochlorite solution and treated with fix and perm cell fixation and cell permeabilization Kit (Life technologies, USA). They were then incubated with anti-5-methyl cytosine monoclonal antibodies for 2 h, washed thrice with PBST (PBS pH 7.4 Tween20 0.1%) and treated with cy3 labelled anti-mouse Rabbit IgGs for 1 h. These embryos were again washed thrice with PBST (PBS pH 7.4 Tween 20 0.1%). Nuclei were stained with DAPI (Promega, USA) and embryos visualized on Axioscope microscope equipped with Axiovision software (Zeiss, Germany).

Statistical analysis

Consistency in methylation signal of each gene in three independent experiments was checked by using one sample *t* test (comparison with mean). The significance of a change between two groups was tested using *t*-test as well as Mann-Whitney U test. The significance of the methylation-expression correlation was assessed using Pearson correlation coefficient. Cluster analysis of methylated genes was carried out using agglomerative hierarchical clustering with Ward's method and a Euclidean distance matrix.

Results

DNA methylation and DNA methyl transferase activity was detected in different developmental stages of *D. melanogaster* ([see Supplementary Information S2 online](#)). After confirmation of presence of DNA methylation in different developmental stages of *D. melanogaster*, methylation microarrays were performed to assess DNA methylation changes in a gene-specific manner.

Methylation pattern in different developmental stages of *D. melanogaster*

Methylation microarray was performed to determine gene-specific methylation in three developmental stages of *D. melanogaster*, viz. adult, pupa and embryo. Out of 12,000 genes present on the *Drosophila* cDNA microarray slide, adult *D. melanogaster* flies showed the presence of methylation in 457 genes in all three biological replicates (*t* test $P < 0.05$) (Table 1, and [see Supplementary Information S3 online](#)). Methylation signal was detected in 373 and 123 genes of the pupa and embryo respectively (*t* test $P < 0.05$) (Table 1 and see [in Supplementary Information S4](#) and [S5 online](#)). The methylated genes specific to each developmental stage, as well as the overlap between different developmental stages are shown in Figure 2 a. The biological process annotations of the methylated genes for the adult, pupa and embryo are shown in Figure 2 b. The genes involved in typical embryonic and larval development processes were found to be methylated in pupal and adult stages of *D. melanogaster*. Similarly, genes that are critically important in pupal and adult development were found methylated in the embryo.

Methylation signature of the adult

Methylation microarray analysis of adult stage identified the presence of consistent methylation in 457 genes (one sample *t*-test $P < 0.05$) ([see Supplementary Information S3 online](#)). Important methylated genes of adult are listed in Table 2. Genes involved in developmental

Table 1. Summary of methylation microarray data

Description	Adult	Pupa	Embryo
Number of genes spotted on <i>Drosophila</i> array	12,000	12,000	12,000
Number of genes positive for methylation signal (All three biological replicates)	970	607	426
Number of genes positive for methylation signal (All three biological replicates with <i>t</i> test $P < 0.05$)	457	373	123
Methylation levels/gene	2.319 (± 1.37)	2.309 (± 1.157)	2.09 (± 1.389)

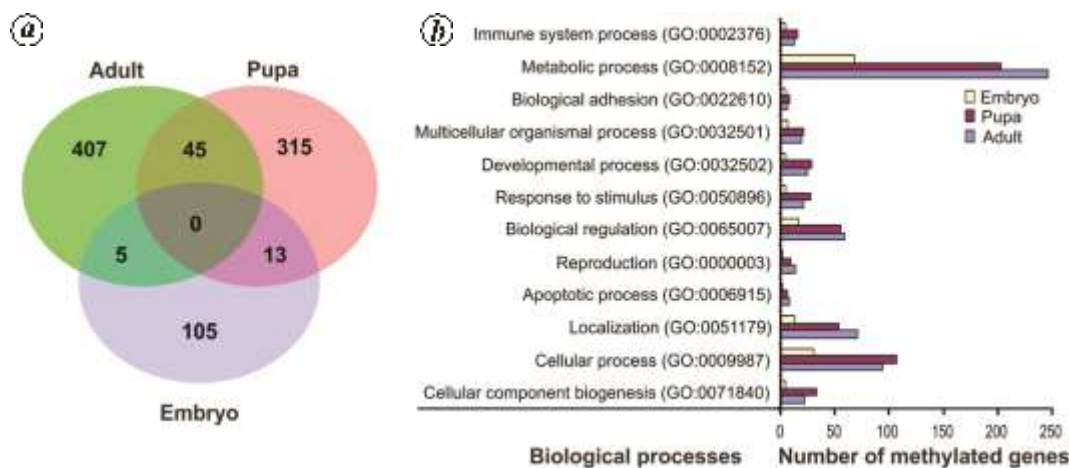


Figure 2. General statistics of probable methylated genes present in different developmental stages of *D. melanogaster*. *a*, Venn diagram of the numbers of overlapping and non-overlapping probable methylated genes in different developmental stages of *D. melanogaster*. *b*, Biological processes of probable methylated genes.

processes, regulation of cell death, negative regulation of signal transduction and negative regulation of Wnt receptor signalling pathway were marked with methylation signal in adult *D. melanogaster*. Furthermore, characteristic embryonic developmental processes such as embryonic pattern specification, oocyte differentiation and development, blastoderm segmentation, anterior/posterior pattern formation and embryonic axis specification showed methylation in adult. Moreover, typical larval developmental processes such as larval locomotory behaviour and imaginal disc-derived appendage and wing development were found to carry cytosine methylation in adults. It is noteworthy that biological processes such as muscle cells, skeletal muscles, organ, muscle tissue, appendage development and regulation of nurse cell apoptosis, which are attributed to pupal development were found methylated in adult (see [Supplementary Information S3 online](#)). Agglomerative hierarchical clustering illustrated four clusters of methylated genes (see [Supplementary Information S6 online](#)). Genes accredited to processes such as chromatin remodelling, gene silencing, tissue development, Wnt signalling and developmental processes were grouped together in cluster 1. Subsequently, genes implicated in processes such as metabolic processes, transport, larval and embryonic development were dominated in cluster 2. Moreover, genes

that have crucial function in cell cycle, stem cell development, cell morphogenesis and phosphorylation were grouped together in cluster 3 and showed moderate methylation. Consequently genes that encode functions involved in metal ion binding and transport across the plasma membrane were marked with higher methylation signal and grouped together in cluster 4.

Methylation pattern of the pupa

Out of 627 methylated genes of pupa, 373 genes showed consistent methylation in all three biological replicates (one sample *t*-test $P < 0.05$) (see [Supplementary Information S4 online](#)). Important methylated genes of pupa are listed in Table 2. Several genes that are known to express in embryonic stages and larval stages showed the presence of methylation in pupa. Genes involved in embryonic development, morphogenesis and differentiation showed evidence of methylation signal in pupa. Moreover, characteristic larval processes such as imaginal disc-derived wing and appendage morphogenesis, instar larval or pupal development and morphogenesis were marked with methylation signal in pupa (see [Supplementary Information S4 online](#)). In addition, genes involved in characteristic adult processes such as gamete generation, sexual

Table 2. Important methylated genes in different developmental stage of *D. melanogaster*

Developmental stage	Important methylated genes
Adult	Frizzled, Knirps-related protein; knrl, trithorax; trx, Caspar, SET domain binding factor; Sbf, Protein hunchback; hb, Bifunctional arginine demethylase and lysyl-hydroxylase PSR, Myc protein; dm; Tumor suppressor protein 101; TSG101, Early girl; elgi, IGF-II mRNA-binding protein, Chromosome associated protein D3; Cap-D3, Fumble; fbl, Enhancer of mRNA-decapping protein 4, Histone RNA hairpin-binding protein; Slbp, folliculin, 5-methylcytosine rRNA methyltransferase nsun4, Centrosomin's beautiful sister; cbs, Protein catecholamines up; Catsup, Probable Bax inhibitor 1, Sterile 20-like kinase, slik, Histone-lysine N-methyltransferase, Disks large 1 tumor suppressor protein; dlg1 and Histone deacetylase complex subunit SAP30
Pupa	Polychaetoid isoform A; pyd, Krueppel target at 95D isoform A; KrT95D, Crammer, Methyltransferase-like protein 13, icln, polycomb protein Su(z) 12, histone-lysine N-methyltransferase trr, Frizzled-4; fz4, Lethal, Wnt inhibitor of Dorsal protein; wntD, ecdysone 20-monooxygenase; shd, Knirps-related protein; knrl, Distracted; dsd and blastoderm-specific protein 25D and nodal homolog
Embryo	N6-adenosine-methyltransferase 70 kDa subunit, developmentally-regulated GTP-binding protein 2, tissue inhibitor of metalloproteases, nipped-b-like protein, dishevelled associated activator of morphogenesis, distracted, histone-lysine n-methyltransferase setd3, polycomb group ring finger protein 3 and pro-epidermal growth factor involved in the epigenetic modification and development
Common in	
Adult-Pupa	Sin1, drl, Tm1, MKP-4, gnu, Lsd-1, knrl, FucT6, Cyp4c3, mura, HDAC6, JhI-21, toy, lilli, vanin-like, Dok, tst, mask, gus, Irp-1A, Dcr-1, myo and mon2
Pupa-embryo	GILT2, Timp, Dyak\GE23254, srl, Rho1, Dsim\GD16174, RpS25, dsd
Adult Embryo	Unc-89, Doa, fbl, l(3)73Ah and Ns3

reproduction and reproductive process in a multicellular organism were found methylated in pupa ([see Supplementary Information S4 online](#)). Agglomerative hierarchical clustering revealed the presence of three clusters ([see Supplementary Information S6 online](#)). Cluster one was dominated by genes involved in typical embryonic and larval development, such as embryonic differentiation and morphogenesis, instar larval development, post-embryonic development and morphogenesis, imaginal disc development, metamorphosis, gastrulation and embryonic development via the syncytial blastoderm. More importantly genes involved in chromatin modification, DNA binding, transcription factors and metabolic processes were found clustered together with development related genes in cluster one. Markedly, genes usually involved in embryonic and larval development and differentiation were grouped together in cluster two. Additionally, genes involved in chromatin modification, DNA binding and transcription were grouped along with development related genes in cluster two. Notably, gene ontology analysis of cluster 3 revealed moderate methylation of genes which play a central role in processes such as metal binding, actin regulation and transport.

Methylation impression of the embryo

Out of 426 genes that showed evidence of methylation signal in the embryo, 123 genes showed consistent methylation (Table 1; one sample *t*-test $P < 0.05$) ([see Supplementary Information S5 online](#)). Important methylated

genes of embryo are listed in Table 2. Gene ontology analysis illustrated that methylated genes are involved in biological processes such as epidermal growth factor receptor signalling pathway, lumen formation, Malpighian tubule morphogenesis, and open tracheal system development. Considering that these processes are not expected to be active in embryo, this stage-specific methylation reveals an interesting pattern. Similarly, genes involved in pathways such as p53 pathway, EGF receptor signalling pathway, gonadotropin releasing hormone receptor pathway, insulin/IGF pathway-mitogen activated protein kinase, FAS signalling pathway, FGF signalling pathway, and toll receptor signalling pathway showed the presence of methylation ([see Supplementary Information S5 online](#)). Noticeably these processes are attributed to pupal and adult stages of *D. melanogaster*. Agglomerative hierarchical clustering of methylated genes showed five clusters and most of the genes were represented in four clusters ([see Supplementary Information S6 online](#)). Cluster 1 was dominated by genes involved in proteolysis, epigenetic regulation of gene expression, actin filament bundle formation and oxidation reduction. Furthermore, genes attributed to processes such as transport, catabolic processes and kinases were grouped together in cluster 2. However, processes involved in oxido-reductase activity, racemase and epimerase activity, translation elongation factor activity, structural constituent of cytoskeleton and sequence-specific DNA binding transcription factor activity showed moderate levels of methylation and clustered together in cluster 3. Moreover, genes involved in isomerase activity, binding,

Table 3. Efficiency of methyl DNA immuno-precipitation of particular genomic loci in different developmental stages of *D. melanogaster*

Developmental stage	Gene	Methylation level by microarray studies	% Methylated DNA-IP/ total input DNA	
			Mean	SD
Embryo	Psc	1.111	3.28E-05	2.74E-05
Embryo	Jhl-26	7.539	0.000744	0.000607
Embryo	Myd88	5.763	5.17E-06	2.45E-06
Embryo	Surf1	1.98	4.21E-06	1.59E-06
Embryo	Rpn3	–	2.50E-06	4.99E-07
Embryo	RpS7	–	ND	ND
Pupa	ida	3.844	0.000115	0.00001
Pupa	opa	3.233	6.01E-05	9.75E-05
Pupa	Bg25D	3.216	0.000432	0.00012
Pupa	Kr-h1	1.779	0.005788	0.000115
Pupa	knrl	1.427	0.001277	0.00055
Pupa	Rpn3	–	2.45E-06	4.95E-07
Pupa	RpS7	–	ND	ND
Pupa	GAPDH	–	ND	ND
Adult	dm	2.325	0.000268	0.000096
Adult	fz	2.271	0.000127	6.77E-05
Adult	grk	5.631	0.072991	0.00963
Adult	knrl	2.872	0.000423	0.000244
Adult	schank	3.872	0.000301301	0.000106
Adult	RpS7	–	ND	ND
Adult	Rpn3	–	2.01E-06	5.95E-07
Adult	GAPDH	–	–	–

ND: not detected.

metallopeptidase activity and structural constituent of ribosome were grouped together in cluster 4.

Methylated genes in different developmental stages

Out of 13 genes that were found methylated in the embryo and pupa, 7 genes demonstrated consistent methylation levels (Mann Whitney *U* test; $P > 0.05$) and 6 genes were differentially methylated (Mann Whitney *U* test; $P < 0.05$). These genes were mainly involved in metabolic processes. Out of five methylated genes that are methylated embryo and adult, four genes showed differential methylation (Mann Whitney *U* test; $P > 0.05$) and were mainly involved in kinase activity. Interestingly, out of 45 genes, genes that are methylated in adult and pupa, 24 genes showed differentially methylated (Mann Whitney *U* test; $P < 0.05$). Heat maps based on methylated status of genes of adult, pupa, embryo, common genes in pupa and adult, common genes embryo and pupa are shown in [Supplementary Information S6 \(see online\)](#). Genes attributed to characteristic embryonic processes such as blastoderm segmentation, embryonic pattern specification, segmentation and pattern specification process were marked with methylation in adult and pupa. Thus methylation of genes in *D. melanogaster* shows distinct life stage specific pattern which shows a functional correlation with their activity states.

Analysis of methylated DNA by MeDIP and q PCR analysis

Selected genes that indicated the presence of methylation were also subjected to MeDIP followed by qPCR to analyse methylation at particular genomic loci. The efficiency of methyl DNA immuno-precipitation for selected genes such as Psc, Jhl-26, Myd88 and Surf1 was higher than Rpn3 and RpS7 genes in the embryos (Table 3). Similarly the efficiency of methyl DNA immuno-precipitation for *ida*, *opa*, *Bg25D*, *knrl* and *Kr-h1* genes was higher than Rpn3 and RpS7 genes of pupa (Table 3). Furthermore, the *dm*, *fz*, *grk*, *Schank* and *Knrl* genes showed higher efficiency of methyl DNA immuno-precipitation than Rpn3 and RpS7 genes of adult *D. melanogaster* (Table 3). This data is consistent with the reported differences in methylation status observed in methylation microarray.

Correlation of methylation status and gene expression values

The methylation-expression correlation in different developmental stages was evaluated by retrieving the expression data available in modMine database (<http://intermine.modencode.org/>). Overall, no correlation between methylation status and gene expression was observed in embryo

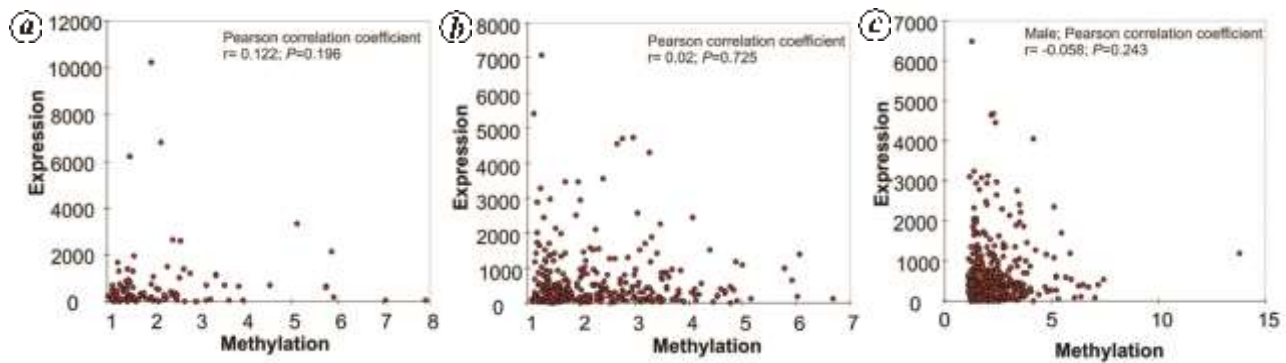





Figure 3. The methylation-expression correlation in different developmental stages was evaluated by retrieving the expression data available in modMine database (<http://intermine.modencode.org/>) for (a) embryo (b) pupa and (c) adults.

Important methylated genes	Adult	Pupae	Embryo
frizzled, Knirps-related protein, trithorax, Caspar, TSG101, Early girl, SET domain binding factor, Protein hunchback, PSR, Myc protein, IGF-II mRNA-binding protein, Cap-D3, Fumble, Slbp, folliculin, dlg1, SAP30 cbs, Probable Bax inhibitor 1, slik, Histone-lysine N-methyltransferase			
	Embryonic pattern specification Blastoderm segmentation Maternal determination of axis	Embryonic development via the Syncytial blastoderm Embryonic morphogenesis Morphogenesis of embryonic epithelium	
	Larval locomotory behavior Imaginal disc-derived appendage wing development morphogenesis	Imaginal disc derived development and morphogenesis, Instar larval development and morphogenesis	Imaginal disc derived development and morphogenesis, Instar larval development and morphogenesis
	Muscle cell and tissue development, Skeletal, striated muscle tissue and organ development, Appendage development, Regulation of nurse cell apoptosis		Lumen formation Tube morphogenesis Malpighian tubule morphogenesis Open tracheal system development
		Female gamete generation Gamete generation Sexual reproduction and reproductive process in a multicellular organism	Oogenesis, gamete generation Reproductive process in a multicellular organism

The characteristic processes of each developmental stage showed presence of methylation in other developmental stages of *D. melanogaster*.

Figure 4. Representative list of methylated genes and characteristic biological processes of each developmental stage of *D. melanogaster* that showed the presence of methylation signal.

(Pearson correlation coefficient = 0.122; $P = 0.196$; Figure 3 a), pupa (Pearson correlation coefficient = 0.02; $P = 0.725$; Figure 3 b) and adults (Pearson correlation coefficient = -0.058 ; $P = 0.243$; Figure 3 c). When correlation in methylation-expression was tested in functionally annotated gene clusters of adults, genes involved in development showed negative correlation with marginally statistically significant P value (Pearson correlation coefficient = -0.226 ; $P = 0.061$). Genes involved in various pathways showed positive correlation in methylation-expression (Pearson correlation coefficient = 0.754;

$P = 0.05$). However, functional annotation clusters of DNA binding (Pearson correlation coefficient = 0.006; $P = 0.964$), apoptosis (Pearson correlation coefficient = 0.343; $P = 0.366$), RNA processing (Pearson correlation coefficient = 0.218; $P = 0.233$) and immunity (Pearson correlation coefficient = -0.576 ; $P = 0.424$) did not show the methylation-expression correlation in adults. Functional annotation clusters of pupa did not show methylation-expression correlations (expression-methylation correlation; Pearson correlation coefficient $P > 0.05$ in all annotation clusters). While in embryo positive correlation

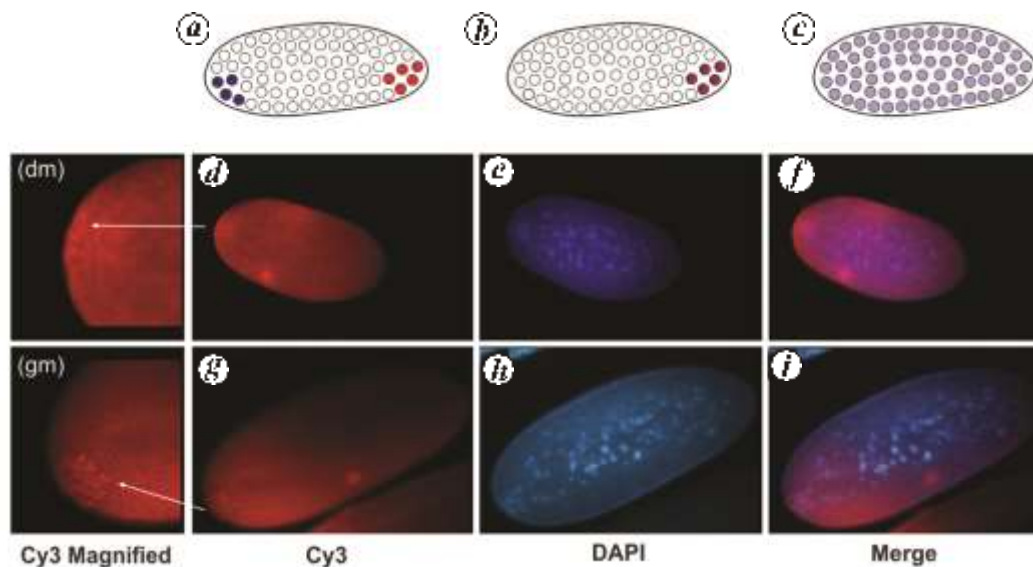


Figure 5. Detection of 5-methyl cytosine in *D. melanogaster* embryos. *a*, *b* and *c* depict the three hypothetical distribution patterns of 5-methyl cytosine in the *D. melanogaster* embryo as suggested by Boffelli *et al.* 5-methyl cytosine was detected in embryos using anti 5-methyl cytosine monoclonal antibodies and visualized by Zeiss AxioScope fluorescence microscope equipped with axiovision software. *d* and *g* depict the presence of 5-methyl cytosine in pole region of embryo. *dm* and *gm* magnified view of *d* and *g*. DAPI stained embryos are shown in *e* and *h*. Cy3 and DAPI merged images are shown in *f* and *i*.

in methylation-expression was observed in proteolytic processes (Pearson correlation coefficient = 0.612; $P = 0.003$), in other functional annotation clusters no significant methylation-expression correlations were observed. These observations suggest that patterns of methylation show significant changes in developmental stage and life cycle related manner.

Discussion

The presence of methylated DNA in several insect species^{5,8,9,39} has directed attention to its potential role in the complex developmental processes of invertebrates. Recently LC-MS/MS and the immuno-precipitation prior to bisulphite conversion studies have reported the presence of DNA methylation in *D. melanogaster* genome^{19,20}. Substantial evidence suggests that immuno-affinity based methods could find application in detecting DNA methylation in *D. melanogaster*^{27,31}. Though earlier studies have identified the methylated regions in *D. melanogaster* genome, a comprehensive list of methylated genes is not reported yet. Tyramide signal amplification (TSA) methods are promising in detection of antigen present in very low amount. TSA methods employ fluorescent dyes to achieve high-resolution signal amplification by at least 100 folds. In the current study, we employed a combination of immuno-affinity and principles of TSA methods to detect methylated DNA in *D. melanogaster*^{27,31}. The binding of a probe to the target via hybridization and detection of methylated cytosine in using immuno-affinity followed by signal amplification

using fluorescently labelled secondary antibodies was used for detection of 5-methyl cytosine. This method was previously employed to identify the methylation profile of the human³², mice³³ and to detect epigenetic alterations in 45, X Turner syndrome³⁴. The present results suggest that this methylation microarray method has promising applications in determining the methylome map. In the current study we exploited methylation microarray to generate the methylome map of different developmental stages of *D. melanogaster*.

In the current study, we describe first the comprehensive list of methylated genes in three life stages of *D. melanogaster*. Immuno-affinity-based detection of 5-methyl cytosine in different developmental stages of *D. melanogaster* suggests the presence of DNA methylation and methyl transferase activity in different developmental stages³⁹. The methylation microarray analysis revealed that adult flies have the highest number of methylated genes (Table 1). Furthermore, MeDIP-qPCR analysis revealed that a small amount of methylated DNA might be present in different developmental stages of *D. melanogaster* (Table 3). The presence of mosaic DNA methylation patterns has been described in different developmental stages of invertebrates^{5,40-44}. DNA methylation increased drastically during developmental stages, and adults exhibited the highest observed DNA methylation level in *Trichinella spiralis*⁴³. It has been demonstrated that DNMT1a is essential during early embryogenesis of *Nasonia vitripennis* and lowering of maternal DNMT1a mRNA resulted in embryonic lethality during the onset of gastrulation⁴⁴. Stable methylation pattern has been

reported throughout the life cycle of Sea Urchin⁴¹. DNA methylation has been shown to affect alternative splicing through its interaction with RNA polymerase II⁴⁵. The present results agree with the earlier studies and suggest that mosaic DNA methylation may exist in *D. melanogaster*.

Insects such as *D. melanogaster* show distinct life stages such as embryo, larva, pupa and adult which are characterized by various characteristic functions. During development, the characteristic functions of each developmental stage are repressed in other developmental stages. The exact mechanisms which control the fine-tuning of molecular mechanisms involved in these developmental stages are not completely understood. Methylation microarray-based analysis showed that the genes involved specialized processes of the embryo, such as embryonic development via the syncytial blastoderm, oocyte differentiation, embryonic pattern specification, axis specification and segmentation showed the presence of methylation in pupa and adult. Similarly, genes that are involved in typical larval processes, such as imaginal disc-derived appendage development, appendage development, and imaginal disc development are found to be methylated in pupae and adults. Interestingly frizzled-4, Krueppel target at 95D isoform A, Wnt inhibitor of dorsal protein and blastoderm-specific protein 25D, trithorax and hunchback, which are the characteristic genes involved in embryonic development were found methylated in pupa and adults. These observations imply that methylation may be involved in fine-tuning of gene regulation processes during the development of *D. Melanogaster* (Figure 4).

It has been documented that one might expect to see negative methylation-expression correlation for methylation located near a gene's transcription start site and positive correlations for methylation located in its body. However, at large-scale no significant methylation-expression correlation (positive or negative) was observed in different developmental stages of *D. melanogaster* (Figure 3). Interestingly, in adults, a negative correlation in methylation-expression was observed in genes involved in developmental processes. Genes involved in various pathways in adult flies showed a positive correlation in methylation-expression. Similar positive correlation in methylation-expression was observed in proteolytic processes of embryo. In both plants and vertebrates, methylation in promoters primarily represses gene expression^{46,47}, whereas methylation in the gene body showed bell-shaped relationship in methylation-expression correlation^{9,30,48,49}. Few processes in *D. melanogaster* follow positive correlation in methylation-expression which is consistent with gene body methylation. Interestingly, development related genes showed negative correlation in methylation-expression suggesting the presence of gene body as well as promoter methylation in *D. melanogaster*. However, in the fungus *Neuro-*

*spora crassa*⁴⁸ and the silkworm *Bombyx mori*¹¹, transcription initiation is not affected by methylation. At large scale, the present results are consistent with earlier results obtained in lower organisms^{11,48}. However, negative and positive correlation in methylation-expression in few functionally enriched clusters suggests the complexity of DNA methylation in *D. melanogaster*. These observations warrant further detailed analysis of methylation and expression correlation in the life stages of *D. melanogaster*. Thus, these results indicate that DNA methylation shows a remarkable diversity in its extent and function across eukaryotic evolution.

D. melanogaster lacks both DNMT1 and DNMT3 genes and has a sparingly methylated genome. The role of DNMT-2 in methylation of *D. melanogaster* has been a matter of considerable debate^{19,20,24,25,50}. It has been suggested that DNMT2 is indeed an RNA methyltransferase that uses a DNA methyltransferase type of mechanism^{24,25}. Given the non-CpG methylation in *D. melanogaster*, asymmetric methylation appears to be more relevant³⁹. Retention of genomic cytosine methylation in Mt2-/- embryos²⁰ raises an interesting possibility of presence of additional methyl transferase activity in *D. melanogaster* which remains to be characterized. LC MS/MS protocol¹⁹ and the combination of methylated DNA enrichment and bisulphite sequencing²⁰ have substantiated the low abundance genomic DNA methylation (less than 1%) in *D. melanogaster*. MeDIP followed by qPCR results suggest less than 1% efficiency of methyl DNA immunoprecipitation of particular genomic loci (Table 3). It has always been suggested that genomic methylation is present in *D. melanogaster* in small quantities and in unusual patterns^{39,50}. Furthermore, it has also been hypothesized that 5-methyl cytosine might be restricted to subset of nuclei⁵⁰. We could detect the distribution of methylated cytosine in pole cells of embryo (Figure 5). Our observations corroborate with restricted distribution of methylated cytosine hypothesis of Boffelli *et al.*⁵⁰. Recently the importance of DNA methylation and Dmmt-2 mediated epigenetic regulation has been shown in *Wolbachia-Drosophila* model system⁵¹. The methylation microarray analysis carried out in the present study provided the list of methylated genes in different life stages of *D. melanogaster*. Our observation of developmental stage-specific modulation of DNA methylation suggests that further analysis is required to ascertain the precise biological role of this DNA methylation in *D. melanogaster*.

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