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***Drosophila* genomic methylation: new evidence and new questions**

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Summary

The presence or absence of 5-methylcytosine in the genome of *Drosophila* has been a matter of disagreement in the literature for decades. We discuss some recent data that have contributed further to the complexity of this issue.

Keywords

DNA methylation; 5-methylcytosine; 5-hydroxymethylcytosine; *Drosophila*

Modification of carbon C5 of cytosine to form 5-methylcytosine (5mC) was one of the first identified epigenetic marks and has been subject to extensive study ever since. 5mC is a major factor in conserved pathways of regulation of gene expression in almost all multicellular organisms. Despite the general role 5mC plays in regulation of vertebrate gene expression, the situation may be different for invertebrates. For example, the nematode *Caenorhabditis elegans* currently presents no evidence for the presence of 5mC and it is widely believed that its genome is devoid of the mark. There is one other potential exception, the model dipteran species *Drosophila melanogaster*. The presence of 5mC in the *Drosophila* genome has been subject to long standing debates from as early as the 1980s with frequent conflicting reports claiming either proof for, or against, the presence of 5mC (1).

As technical abilities have advanced so has the debate. For example, Lyko et al. reported in the year 2000 that methylation was present on *Drosophila* DNA but it was restricted to early embryo development (2). This was supported by a report a few years later showing immunostaining of 5mC in embryonic DNA from the same time points (3). More recently, early generation site-specific bisulfite sequencing was used to show that the presence of 5mC was associated with the silencing of retrotransposons and telomere integrity (4). There has been much debate surrounding these articles and their specificity with Raddatz et al. using whole genome bisulfite sequencing to come to the conclusion that “*Drosophila melanogaster* lacks detectable DNA methylation patterns” (5). The reason for this controversy derives not just from the conflicting data but also the apparent lack of orthologs

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Conflicts of interest:

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within *D. melanogaster* of the central epigenetic enzymes responsible for the establishment and maintenance of the 5mC mark in mammals.

The human genome contains three well studied DNA methyltransferase enzymes responsible for establishing (DNMT3a/3b) and maintaining (DNMT1) the genomic 5mC epigenetic mark as well as three members of the ten-eleven translocation (*TET*) family of Fe²⁺- and 2-oxoglutarate-dependent dioxygenases (*TET*1/2/3) recently found to be responsible for the progressive oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and finally to 5-carboxylcytosine (5caC) (6). There is also an enzyme identified by homology to the DNMT methyltransferases, DNMT2 (7), which is now felt to be responsible for the methylation of the cytosine at position 38 in tRNA^{Asp} (8), though activity as a DNA methyltransferase has not been completely ruled out.

Certain insects such as honeybees (*Apis mellifera*) contain orthologs to both DNMT1 and DNMT3, which have been shown to play a role in the generation of different phenotypes in a bee colony via different genomic 5mC patterns (9,10). The genome of *D. melanogaster*, as well as seemingly the whole order of *Diptera*, varies significantly from that of humans, honeybees and other insects, in that it only contains a single recognised DNMT ortholog of DNMT2 (Mt2) which means there is no apparent method for establishing de novo 5mC marks. *D. melanogaster* also has a single ortholog of the *TET* family proteins which in humans are known to use 5mC as a substrate (1). These are somewhat conflicting ideas, an enzyme that uses 5mC as a substrate, but no enzyme to make that substrate? As mentioned previously, this discussion of 5mC has evolved as new techniques became available. In the last few months, three new reports have appeared which shed further light on this apparent epigenetic conundrum.

The first of these recent reports used early stage embryos, the same time points as used by Lyko et al. (2) and employed “MeDIP-Bseq” which couples antibody based enrichment of 5mC-containing fragmented genomic DNA with bisulfite treatment and Illumina sequencing in an attempt to achieve a deeper coverage of specifically methylated regions of the embryo genome (11). With this method the authors confidently detected 5mC genome coverage of 0.01% of cytosine. They also compared oocytes with stage 5 embryos and saw distinct differences in methylation patterns; these development stage differences are not likely to be seen with non-specific 5mC signals. Interestingly, they also found that methylation is present on only a subset of alleles in the embryos, a finding which gives rise to the possibility that 5mC is only used in certain loci in certain cell types or lineages and may therefore be used in defining cellular developmental pathways. To confirm that the only recognised candidate for 5mC production, DNMT2/Mt2, was producing the detected 5mC they examined Mt2 null *Drosophila* but found no effect on 5mC levels. This is somewhat surprising and conflicts with earlier reports indicating that Mt2 was responsible for production of detected 5mC. This newer study would imply the existence of a novel, hereto unidentified, methyltransferase enzyme.

The second report also seems strongly to support the presence of 5mC in the *Drosophila* genome. Using an LC-MS/MS method and “state of the art” HPLC equipment coupled to a triple quadrupole mass spectrometer, Capuano et al. were able clearly to detect 5mC in

samples of whole adult *Drosophila* (12). This result supports the previously mentioned work, but both of these reports seem to conflict with evidence from recent whole genome bisulfite sequencing. The authors state their measurements gave a 5mC level of about 0.034% of C (12). This level would be below the error rate for bisulfite sequencing of ~0.5%, so potentially this low level of methylation would be missed in conventional bisulfite sequencing. It is worthy of note that both of these reports estimate similar total 5mC levels, and when one considers the possibility that MeDIP-Bseq may have missed 5mC in regions that cannot be uniquely mapped, the likelihood of the levels being the same rises lending greater support to the validity of these data.

We recently presented evidence for the presence of 5mC in *Drosophila* based on the occurrence of dTet, a well-conserved member of the TET 5mC dioxygenases family in *Drosophila* (1). The strength of evidence for the presence of 5mC discussed above now seems to outweigh the evidence for absence and the recent findings now immediately suggests the logical presence of dTet in the fly genome. A very recent report, however, revealed that the mammalian TET family of proteins is capable of using RNA as a substrate, though with a considerably lower affinity as DNA (13). Also, 5hmC is the final product of the reaction in RNA, in contrast to 5caC, the final DNA oxidation product. The report shows that when over-expressed in vivo, only a single member of the TET family proteins, TET3, is capable of producing the 5hmC nucleoside (13). Previously we showed data indicating that TET3 was the TET enzyme most similar to the TET protein found in *Drosophila* (1). These data combined could have been used to explain the presence of dTet as an RNA-modifying enzyme, but the recent higher sensitivity 5mC data (11,12) means that a final determination is not currently possible.

One aspect of this problem that remains unresolved is the possible presence of 5hmC in *Drosophila*. Given the strong evidence for the presence of genomic 5mC and the dTet protein it is expected that 5hmC would be present. In mice the highest levels of 5hmC are found in the brain along with Tet3 being highly expressed in developing brain (14,15). This finding lends supports to the expectation of 5hmC being present, which might also correlate with dTet expression patterns where the highest levels are found in the central nervous system and the brain (1). It is also worth noting that 5hmC is detected at about 10-20% the level of 5mC in human brain at 0.5-1% of all DNA cytosine. With 5mC levels reportedly between 0.01% and 0.034% of cytosine in *Drosophila*, the mammalian systems would indicate that 5hmC levels could be as low as 0.001% of cytosine when utilising whole embryos, or whole adults. These low levels would require the highest sensitivity methods to detect. As mentioned, 5mC levels are seemingly variable between alleles and potentially determined by cell type. If this is the case it leads to the possibility that certain cell types/lineages could have substantially more 5mC than the reported averages. Given that dTet expression is clearly detectable by stage 16 (15 h post fertilisation) and is seemingly limited to both the brain and central nervous system it will be important to limit searches for 5hmC to those tissues where dTet expression is the highest to allow for easier detection (1).

However, the data discussed above leads to some other interesting conclusions, which can be made by taking into account the sensitivity of the latest methylation data. The first is: what is the source of the 5mC generation? The methylation could potentially be created by

Mt2, but with no changes in 5mC seen upon Mt2 deletion, it would imply there are one or more novel DNA cytosine methyltransferases in *Drosophila*. It may not have been an obvious expectation that members of the TET family could act on DNA and RNA, but given evidence for this ability, is it now logical to reverse expectation and predict that one of the RNA methyltransferases in *Drosophila* could have evolved to act upon DNA? There is not currently any evidence of this, but it is an interesting possibility. The second conclusion is that given the finding that TET family members are found almost ubiquitously throughout metazoans from the early ctenophore (*Pleurobrachia bachei*) to human (16), it appears likely their potential new dual function as both DNA/RNA 5mC oxidisers would have developed early in evolution and therefore can be expected to play a vital role in both gene regulation and in RNA stability/activity. Due to the very recent identification of 5hmC in mammalian RNA the role played by this modification has still to be elucidated, but it is likely to be important given its conservation during metazoan evolution. Finally, given the apparent conserved expression patterns of TET proteins from very diverse organisms, the presence of 5hmC could be important in regulating the correct development of brain and central nervous systems.

In conclusion, evidence is gathering in favour of the fact that there is methylation in *Drosophila* though its source is still elusive. With the rapid progress of research outlined above it is expected that evidence will continue to accumulate and will eventually allow a final answer to the question 'Is there methylation in the *Drosophila* genome?' It is impossible to be absolutely certain at this stage as to what the answer will be, but the path leading there is expected to provide an exciting journey.

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