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SYMPOSIUM

DNA Methylation in Basal Metazoans: Insights from Ctenophores

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Synopsis Epigenetic modifications control gene expression without altering the primary DNA sequence. However, little is known about DNA methylation in invertebrates and its evolution. Here, we characterize two types of genomic DNA methylation in ctenophores, 5-methyl cytosine (5-mC) and the unconventional form of methylation 6-methyl adenine (6-mA). Using both bisulfite sequencing and an ELISA-based colorimetric assay, we experimentally confirmed the presence of 5-mC DNA methylation in ctenophores. In contrast to other invertebrates studied, *Mnemiopsis leidyi* has lower levels of genome-wide 5-mC methylation, but higher levels of 5-mC methylation in promoters when compared with gene bodies. Phylogenetic analysis showed that ctenophores have distinct forms of DNA methyltransferase 1 (DNMT1); the zf-CXXC domain type, which localized DNMT1 to CpG sites, and is a metazoan specific innovation. We also show that ctenophores encode the full repertoire of putative enzymes for 6-mA DNA methylation, and these genes are expressed in the aboral organ of *Mnemiopsis*. Using an ELISA-based colorimetric assay, we experimentally confirmed the presence of 6-mA methylation in the genomes of three different species of ctenophores, *M. leidyi*, *Beroe abyssicola*, and *Pleurobrachia bachei*. The functional role of this novel epigenomic mark is currently unknown. In summary, despite their compact genomes, there is a wide variety of epigenomic mechanisms employed by basal metazoans that provide novel insights into the evolutionary origins of biological novelties.

Introduction

Epigenomics is an emerging field that studies the functional elements that define the regulation of gene expression in a cell. The data provided by the NIH Roadmap Epigenomics Consortium demonstrate the extreme complexity of the human epigenome and that a wide variety of non-mendelian, reversible modifications of DNA can effect gene transcription (Kundaje et al. 2015). Methylation and accessibility of DNA, chromatin states, and modifications and replacements of histones have been extensively studied in humans and in a few model organisms (Bogdanovic 2014), but little is known about the presence and diversity of these mechanisms across the animal kingdom. Here, we discuss DNA methylation in non-bilaterian basal metazoans. Surprisingly, besides the conventional form of metazoan genomic DNA methylation, 5-methyl cytosine (5-mC), we detected a second type of genomic DNA methylation: formation of 6-methyl adenine (6-mA) in ctenophores. Recent phylogenetic analysis strongly suggests the placement of ctenophores as a sister group to all other metazoans (Ryan et al. 2013; Moroz et al 2014; Borowiec et al. 2015; Whelan et al. 2015a, 2015b) with a possibility that the ctenophore lineage independently evolved many metazoan characteristics such as neurons, muscles, and mesoderm (Moroz 2014, 2015; Moroz et al. 2014). The discovery of two DNA methylation mechanisms in ctenophores opens novel opportunities to study their functional role in development, regeneration, and neural organization as well as the evolution of DNA methylation in metazoans. In this article, we first briefly review the

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comparative literature related to the evolution of DNA methylation. Second, we then provide experimental analysis of two mechanisms of DNA methylation in ctenophores. Finally, we discussed the obtained data within the context of the evolution of the mechanisms of DNA methylation across major metazoan lineages.

Ubiquitous features of DNA methylation

DNA methylation occurs throughout all domains of life from Eubacteria and Archaea, to plants, and metazoans (Rojas and Galanti 1991; Henderson and Jacobsen 2007; Harony and Ankri 2008; Iyer et al. 2011; Capuano et al. 2014). In eukaryotes, cytosine methylation is the most common modification of DNA in which a methyl group is covalently added to the fifth carbon of the cytosine on the pyrimidine ring to form 5-methyl cytosine (5-mC) (Bird 2002, 2007). These cytosines are often located upstream (5′) of guanines as part of a CpG dinucleotide, but non-CpG sites such as CpHpG (in which an H is A, T, or C) and nonsymmetrical CpA and CpT are also methylated at a lower frequency (Ichiyanagi et al. 2013).

In prokaryotes, DNA methylation occurs both on cytosine and on adenine bases. It is a part of the bacterial host's defense system that protects the bacteria against infection by bacteriophages (Noyer-Weidner and Trautner 1993; Bird et al. 1995; Jeltsch 2002; Zemach et al. 2010; Zemach and Zilberman 2010). In plants, cytosine methylation occurs throughout all sequence contexts and is involved in gene-silencing as well as in unique transposon-silencing (Henderson and Jacobsen 2007; Lister et al 2008).

In bilaterians, germ cells from both parents undergo epigenomic reprogramming when somatic methylation patterns are erased and sex-specific and gamete-specific DNA methylation patterns are added (Morgan et al. 2005; Drewell et al. 2014; Olson and Roberts 2014). During the first stages of embryogenesis the pattern of DNA methylation from one or both parental alleles undergoes de-methylation and the embryo establishes its own pattern of DNA methylation as it differentiates (Jaenisch et al. 1982; Jahner et al. 1982; Stewart et al. 1982; Jiang et al. 2013; Riviere et al. 2013; Messerschmidt et al. 2014). Despite this erasure, certain patterns of DNA methylation are still heritable due to epigenetic imprinting, although the exact mechanism of this inheritance is not clear (Morgan et al. 2005; Saitou et al. 2012).

In most cases, the 5-mC DNA methylation of vertebrates occurs within the context of a CpG dinucleotide with 60–90% of CpG sites being methylated (Ehrlich et al. 1982; Tucker 2001; Lister and Ecker 2009; Lister et al. 2009). As such, CpG dinucleotides occur with a much lower frequency in the genomes of vertebrates than would be expected due to chance. The frequency of CpG dinucleotides in the human genome is only 1%, i.e., less than one-quarter of the expected frequency (Rein et al. 1998; Zilberman and Henikoff 2007).

The presence of DNA methylation in the genomes of invertebrates was initially controversial because the two model organisms, *Drosophila melanogaster* and *Caenorhabditis elegans*, are mostly free of 5-mC methylation (Bird et al. 1995). Subsequent comparative studies using representatives from several other metazoan lineages showed 5-mC DNA methylation being present in their genomes (Tweedie et al. 1997; Zemach et al. 2010; Mendizabal et al 2014). DNA methylation is already proving to be a pivotal regulator of development in honey bees and oysters (Harony and Ankri 2008; Riviere et al. 2013; Drewell et al. 2014). To date, studies on methylation in invertebrates have only been performed for a relatively small number of invertebrate species, the majority of which are arthropods (Supplementary Table S1). Including our current study, even fewer have had their genome-wide methylation patterns analyzed with single base resolution (Supplementary Table S1). Overall, the invertebrates studied so far have a lower percentage of their CpG sites methylated than do mammals but, consistent with mammals, all analyzed species have higher amounts of gene-body methylation than promoter methylation (Su et al. 2011). Here, we performed a comparative survey of enzymes responsible for DNA methylation across metazoans focusing on ctenophores.

Diversity of DNMTs

DNMTs are a family of enzymes that catalyze the transfer of a methyl group to cytosine in DNA with specialized methylase enzymatic domains (Bird 2002, 2007; Iyer et al. 2011). Broadly, metazoan DNMTs enzymes fall into two main groups: DNMT1 and DNMT3 (Iyer et al. 2011; Jurkowski and Jeltsch 2011). In chordates, these two enzymes play different roles (Jia et al. 2007). The DNMT1 enzyme is involved in maintenance of methylation during cell division while *de novo* DNA methylation during embryonic development is thought to be performed by the DNMT3 enzyme because of its ability to bind to both hemi-methylated and unmethylated

CpG sites with equal affinity (Kangaspeska et al. 2008; Holz-Schietinger et al. 2011). Knocking out both alleles of these *DNMT* genes has proved to be lethal to embryos, thereby demonstrating that the *DNMT1* and *DNMT3* genes are both necessary for cell differentiation in vertebrates (Dhe-Paganon et al. 2011; Li et al. 1992). DNMT1-like sequences have been found in all metazoan phyla, including ctenophores, with the exception of Placozoa. The two ecdysozoan lineages leading to *D. melanogaster* and *C. elegans* have lost both *DNMT* genes, thereby accounting for their lack of 5-mC DNA methylation (Yi 2012).

Structural organization of DNMT

In humans there is one *DNMT1* gene with at least four isoforms localized to chromosome 19 (el-Deiry et al. 1991). The *DNMT1* (NM_175629.2) of humans consists of 41 coding exons with several identified transcription–initiation sites (Bigey et al. 2000) (Fig. 1). Interestingly, the *DNMT1* of ctenophores (Moroz et al. 2014) is much larger than arthropods' *DNMT1* gene, even though its genome is half the size of the honey bee's genome, *Apis mellifera* (Weinstock et al. 2006). The *DNMT1* of *Apis* (XM_006562802.1) has 15 coding exons whereas *DNMT1*-like (JX985458.1) gene of *Pleurobrachia bachei* has 29 exons (Fig. 1).

Structurally, the DNMT1 enzyme has many protein domains that appear to be conserved across all Metazoa (Goll and Bestor 2005; Denis et al. 2011). In mammals, starting at the N-terminus is a domain for secondary protein interaction such as the DNA-methyltransferase-associated protein (DMAP) that binds to DNMT1 and recruits histone deacetylases to facilitate the formation of heterochromatin (Goll and Bestor 2005; Song et al. 2012). Next, the replication foci domain (RFD) contains a target-recognition sequence that localizes the enzyme to replication forks, followed by a zinc finger with a C-X-X-C domain to recognize CpG sites, and 2-bromo-adjacent homology (BAH) domains that allow protein–protein interactions. The enzymatic DNA-methylase (PF00145) domain is located at the C-terminus (Fig. 1) (Song et al. 2012). The crystal structure of the DNMT1 enzyme complexed with DNA has also been resolved showing it does bind to DNA (Song et al. 2011) (Fig. 1). Based on published sequences, the DMAP domain appears to be conserved only in chordates (Albalat 2008). Outside of Metazoa, DNMT1-like proteins have no CXXC domain and the presence and number of RFD and BAH domains vary greatly (Iyer et al. 2011) (Fig. 5).

There are three *DNMT3* genes (*DNMT3A*, *DNMT3B*, and *DNMTL*) that each have numerous isoforms in humans (Yanagisawa et al. 2002). DNMT3 genes are located on different chromosomes; *DNMT3A*, *DNMT3B*, and *DNMTL* are on chromosomes, 2, 20, and 21, respectively.

The DNMT3 enzyme is defined in metazoans by the presence of the PWWP motif, involved in protein–protein interactions, the ADDz domain, a PHD-like zinc finger domain that binds to the histone H3 tail at unmethylated lysine 4, and all DNMT3s have a single DNA-methylase domain at the c-terminus. The crystal structure has been determined for *DNMT3A* complexed with *DNMT3L* (Jia et al. 2007) (Fig. 1). It is shown that even though DNMT3L cannot methylate DNA *de novo*, it acts as a regulatory subunit for the DNMT3A gene during maternal genomic imprints (Jia et al. 2007). The DNMT3 protein of *Apis* has a domain organization similar to that of humans (Fig. 1). No genome or transcriptome of ctenophores that have been analyzed so far contains a *DNMT3* gene.

In metazoans, the DNMT2 that has a cytosine-specific-methylase domain has been show actually not to be a DNMT, but rather a tRNA aspartic acid methyltransferase 1 and thus was renamed TRDMT1 (Goll et al. 2006). In all other non-metazoan eukaryotes, DNMT2-like proteins are also tRNA-specific methyltansferases with the exception of a parasitic unicellular eukaryote, *Entamoeba histolytica*, that has a DNMT2-like enzyme that can perform both DNA and tRNA methylation (Tovy and Ankri 2010). Our analysis indicates that the genomes of the ctenophores *Pleurobrachia* and *Mnemiopsis* each contain a single *TRDMT1* gene, which shares highest identity to metazoan TRDMT1-like enzymes.

Mosaic nature of DNMT enzymes

DNMT-type enzymes in non-metazoans

Bacteria as well as Archaea have enzymes that methylate DNA at different bases and contexts (Iyer et al. 2011). The prokaryotic DNMTs target either cytosines or adenines for methylation. These prokaryotic enzymes are parts of restriction–modification systems responsible for the methylation of hosts' DNA sequences for protection (Iyer et al. 2011).

Interestingly, recent analysis of the eukaryotic genomes of *Drosophila*, *Caenorhabditis*, and *Chlamydomonas reinhardtii* confirmed the presence of 6-mA DNA methylation and the enzymatic machinery that performs this methylation (Fu et al. 2015; Greer et al. 2015; Zhang et al. 2015).

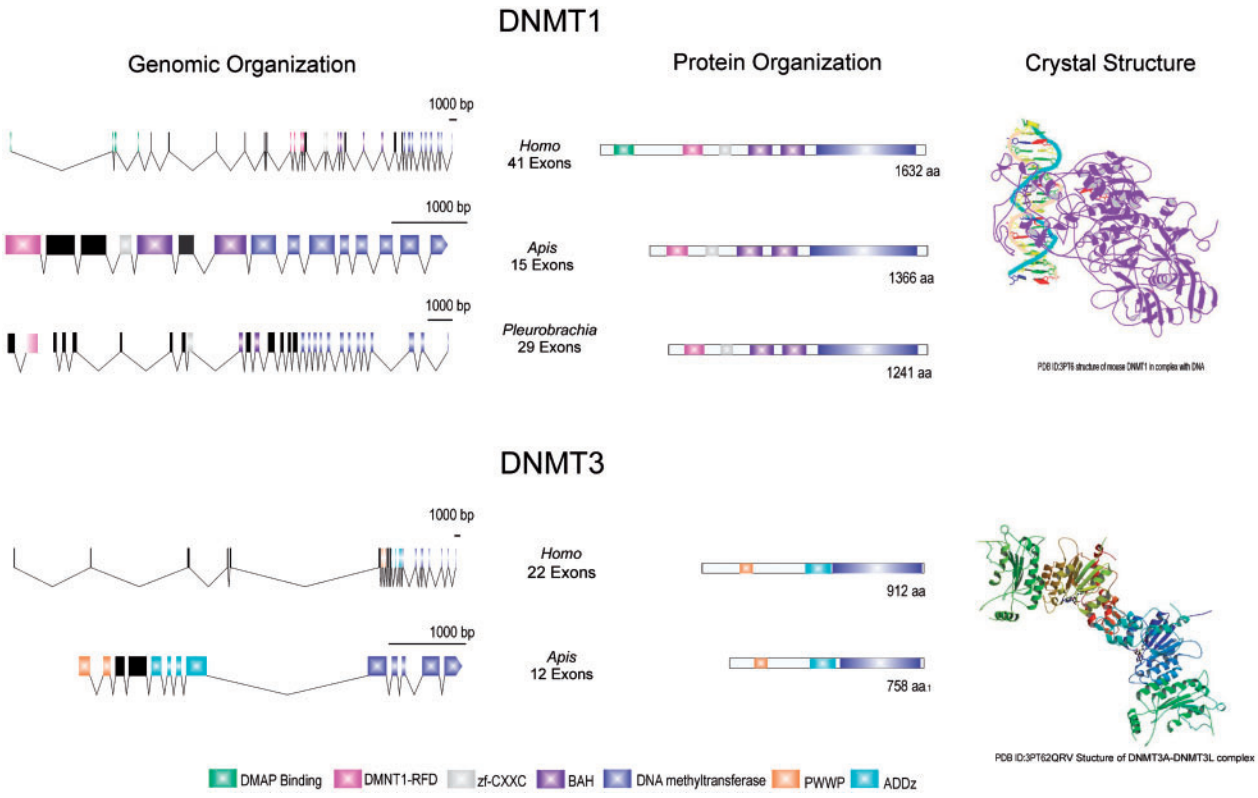


Fig. 1 Structural organization of DNMT enzymes. *Homo DNMT1* (NM_175629.2) gene consists of 41 coding exons and is 61,734 bp long. The *Apis mellifera* DNMT1 (XM_006562802.1) gene has 15 coding exons and is 6183 bp long. The *Pleurobrachia* DNMT1-like gene (JX985458.1) has 29 exons and is at least 16,598 bp long (located on two scaffolds). The protein structure of the DNMT1 of humans is comprised of a DNA methyltransferase-associated protein-binding domain (DMAP), replication foci domain (RFD), C-X-X-C domain, 2 Bromo-adjacent homology (BAH) domains, and DNA-methylase (PF00145) domain. The predicted DNMT1 proteins of *Apis* and *Pleurobrachia* contain the same domains but each lack a DMAP domain. Crystal structure of mouse DNMT1 (650–1602) in complex with DNA is shown [PDB ID: 3PT6] (Song et al. 2011). **Structural organization of the DNMT3 enzyme**. There are three DNMT3 genes in humans and we use the DNMT3A as an example. The *Homo* DNMT3A (NM_175629.2) gene consists of 22 coding exons and is 109,629 bp long. The *Apis mellifera DNMT3* (XM_006562802.1) gene has 12 coding exons and is 43,394 bp in length. The DNMT3 enzyme is defined by the presence of the PWWP motif known to be involved in protein–protein interactions with, ADDz domain, a PHD-like zinc-finger domain, and a DNA methylase domain. The crystal structure for DNMT3A complexed with DNMT3L is shown [PDB ID: 3PT62QRV] (Jia et al. 2007). No DNMT3s were found in the sequenced genomes or transcriptomes of ctenophores (Moroz et al. 2014). (This figure is available in black and white in print and in color at *Integrative and Comparative Biology online*)

DNMT enzymes present in bilaterians

The majority of sequenced bilaterian genomes appear to have at least one *DNMT1* gene with few exceptions, mostly in dipteran insects and nematodes (Marhold et al. 2004; Yi 2012). However, although present in most metazoans, there have been several losses of DNMT3, particularly in protostomes. For example, there is a *DNMT1* gene present in the gastropod mollusc *Aplysia californica*, but no *DNMT3* gene has been detected. Yet, in the gastropod mollusc *Lottia gigantea* as well as in the bivalve *Crassostrea gigas* the genomes contain both *DNMT1* and *DNMT3* genes (Wang et al. 2014). A mosaic of DNMT3 gene losses or duplications is also observed in insects (Glastad et al. 2014).

DNMT enzymes present in non-bilaterian basal metazoans

All sequenced non-bilaterian basal metazoans have the *DNMT1* gene with the exception of *Trichoplax adhaerens*. *Trichoplax* also does not have a *DNMT3* gene, thereby suggesting the absence of canonical 5-mC type methylation in its genome. However, the *DNMT3* gene is present in at least one species of cnidarian (*Nematostella vectensis*) and one poriferan (*Amphimedon queenslandica*) (Lyko and Maleszka 2011). Two sequenced genomes and six transcriptomes of ctenophores (Moroz et al. 2014) contain only one *DNMT1* gene or its corresponding mRNA (Figs. 1 and 2). No *DNMT3* gene was detected in the genomes of either *Pleurobrachia* or *Mnemiopsis*, or in

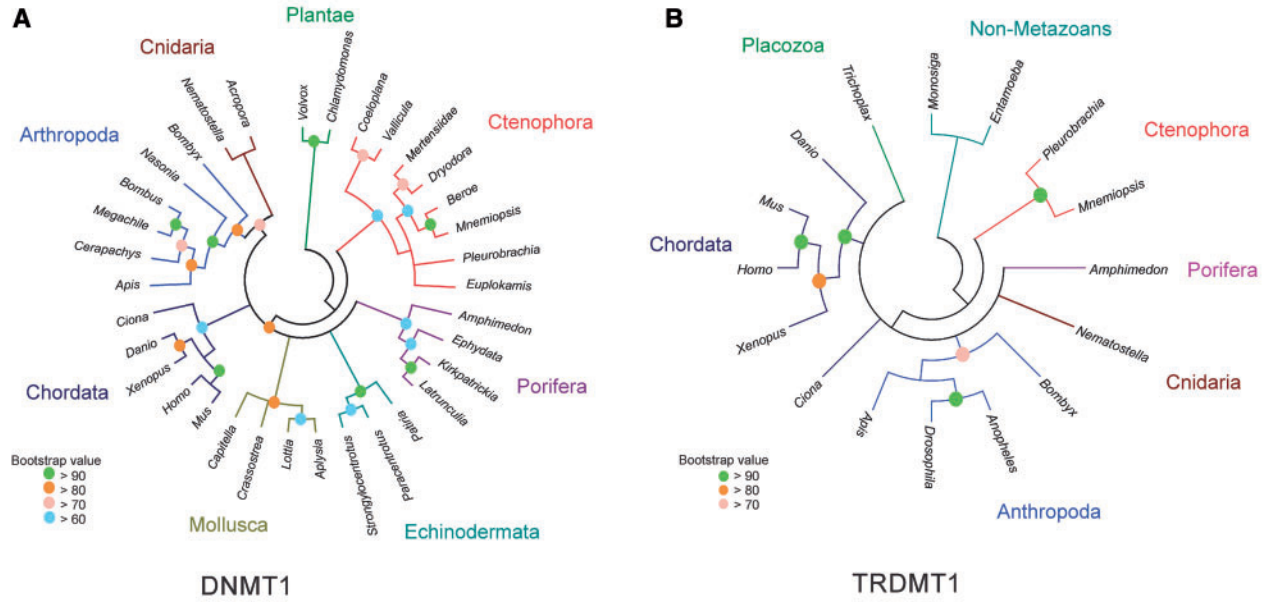


Fig. 2 Genealogical relationships of DNMTs. (**A**) Phylogenetic analysis of DNMT1 was performed by constructing a maximum likelihood using the methyltransferase domains from DNMT1 sequences. All branches with bootstrap support less than 50 have been collapsed. Percent Bootstrap values are indicated by dots. This tree was rooted to the know outgroup sequences from chlorophycean algae *Volvox carteri* and *Chlamydomonas reinhardtii*. Within this clade the benthic ctenophores *Vallicula multiformis* and *Coeloplana astericola* cluster together. (**B**) Phylogenetic analysis of TRDMT1 by constructing a maximum likelihood using the methyltranferase domains from DNMT1 sequences. All branches with bootstrap support less than 50 have been collapsed. Percent bootstrap values are indicated by dots. This tree was rooted to known non-metazoan outgroups the choanoflagellate *Monosiga brevicollis* and the amoebozoan *Entamoeba histolytica*. Both of these trees demonstrate the basal branching of ctenophore DNMT family sequences. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology online*)

the other transcriptomes of ctenophores that were analyzed.

The *Pleurobrachia* DNMT1-like sequence (AFV53350.1) shares the highest identity with metazoan DNMT1 sequences (approximately 75% identity) (Moroz et al. 2014). We also identified partial sequences of *DNMT1*-like genes in the genome of *Mnemiopsis* and in the transcriptomes of six other species of ctenophore as well.

Parallel evolution of DNMTs

The ancestral origins of *DNMT1*, *DNMT3*, and *TRDMT1* genes

TRDMT1 contains only a methyltransferase domain that shares high identity with DNMT domains; however, its methylase activity is specific for RNA (Goll and Bestor 2005). Extensive phylogenetic and 3D BLAST clustering analysis of DNMT1, DNMT3, TRDMT1, and RNA methyltransferases demonstrated that TRDMT1 is a part of the DNMT family. It is thought that TRDMT1 changed its functional role to RNA-specific methylation at some early stage in the evolution of eukaryotes; although one example of a prokaryotic TRDMT1 acting as an RNA methyltransferase has now been discovered (Jurkowski and Jeltsch 2011; Shanmugam et al. 2014). The eukaryotic TRDMT1 shares high identity with the prokaryotic TRDMT1-like enzymes. It was initially postulated that the TRDMT1 was the ancestor of DNMT1 and DNMT3 (Goll et al. 2006). However, Jurkowski and Jeltsch (2011) suggested that eukaryotic DNMT1 or DNMT3 and TRDMT1 enzymes all have had independent origins (Jurkowski and Jeltsch 2011). Our phylogenetic analysis shows that ctenophore TRDMT1-like sequences cluster with metazoan TRDMT1-like sequences (Fig. 2B).

Genealogy of DNMT-like enzymes in metazoans

We tested the genealogical relationship of metazoan DNMT1 and DNMT1-like enzymes. Lineage-specific clustering and radiation were evident for representatives of all phyla analyzed, including newly identified poriferan DNMT1-like sequences (Fig. 2). We used DNMT1-like sequences from algal species for a metazoan outgroup, and as such constructed our phylogeny using only the conserved methyltransferase protein domain. This analysis demonstrates that the ctenophore DNMT1 gene family branched close to the base of the metazoan cluster.

Within the ctenophore-specific subcluster, a sister relationship was noticed between the two DNMT1

enzymes from two benthic ctenophores, *Coeloplana astericola* and *Vallicula multiformis* (Fig. 2A); their longer branches also suggest relatively faster evolutionary changes within this group. We refer to all of these predicted genes as "like" since these DNMT1-like sequences have not been expressed or characterized.

We also surveyed possible relationships of ctenophore TRDMT1 sequences to other metazoans. Specifically, we aligned the methyltranferase domains and used known eukaryotic TRDMT1 sequences as an outgroup. The reconstructed genealogical trees suggest that ctenophore TRDMT1 sequences are quite distinct (Fig. 2B), but the base of the tree could not be resolved.

Genealogy of all DNMT enzymes across plants, fungi, unicellular eukaryotes, and metazoans

Next, we tested the genealogical relationships among different classes of DNMT enzymes at the larger phylogenetic scale (Supplementary Fig. S1). The gray area in Supplementary Fig. S1 is the same as the DNMT1 tree shown in Fig. 2. The red branches indicate the cluster of ctenophores' DNMT1-like sequences (Supplementary Fig. S1).

The genealogical relationship reveals three main branches for DNMT1-like, DNMT3-like, and TRDMT1-like enzymes, respectively (Supplementary Fig. S1). However, the addition of plants, fungi, unicellular eukaryotes, and even bacteria cause the analysis to become unstable, thus producing low resolution at these nodes, which is consistent with the low support seen on most extensive DNMT trees in the literature (Supplementary Fig. S1) (Iyer et al. 2011; Jurkowski and Jeltsch 2011).

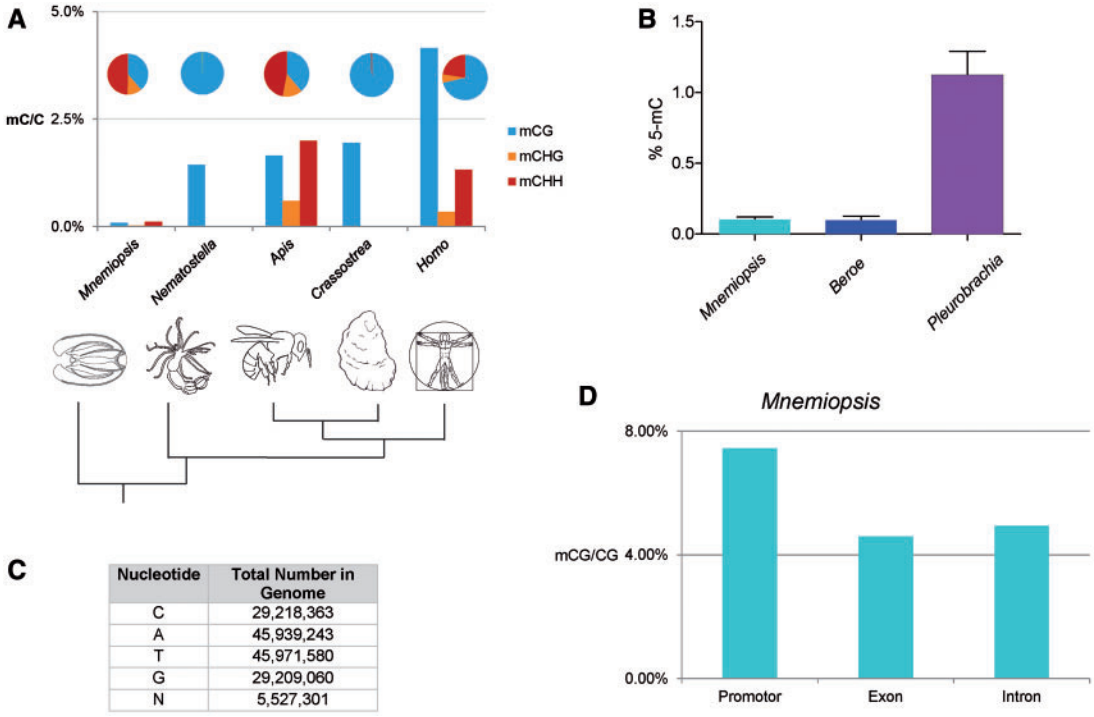
Global 5-mC DNA methylation

Several organisms such as yeast, *Saccharomyces cerevisiae*, and the nematode *C. elegans* have no canonical DNMTs, and are devoid of detected cytosine methylation in their genomes (Bird et al. 1995). The genomes of *D. melanogaster* and several other dipteran insects contain a *TRDMT1* gene that produces a nonfunctional enzyme with negligible levels of cytosine methylation (Marhold et al. 2004; Lyko et al. 2006). However, this is not the case in all insects, as the honey bee, *A. mellifera* was shown to have a fully functional DNA 5-cytosine methylation system (Fig. 1) (Wang et al. 2006). Global cytosine methylation has also been measured in other Hymenoptera including ants, bees, and wasps (Kronforst et al. 2008) (Supplementary Table S1). The invertebrates that have been studied overall have lower levels of methylation than the levels observed in mammals (Fig. 3A). Yet, initial experiments on insects and molluscs have demonstrated the functional necessity of DNA methylation for regulating development and memory (Kucharski et al. 2008; Lockett et al. 2010; Gavery and Roberts 2014; Lukowiak et al. 2014).

Among four lineages of non-bilaterian basal metazoans, only the methylome of the cnidarian *N. vectensis* has been characterized, although no functional testing on the role DNA methylation plays has been performed (Zemach et al. 2010).

Genome-wide analysis with ELISA-based colorimetric assays showed approximately 0.5–1% of the genome of the ctenophore *Pleurobrachia* to be methylated (Moroz et al. 2014). Quantitative transcriptome profiling across all major developmental stages and across tissues of adult *Pleurobrachia* also reveal patterns of mosaic expression of its DNMT1, DNA-methylation enzyme, as well as the demethylation enzyme, TET (Moroz et al. 2014). We performed further ELISA assays for 5-mC methylation on *Mnemiopsis leidyi* and *Beroe abyssicola*, and compared them with samples from *Pleurobrachia* gDNA within the same assay, and found approximately 0.11%, 0.12%, and 0.8–1.2% methylation, respectively (Fig. 3B).

Although there are many methods for measuring DNA methylation, only bisulfite conversion followed by sequencing allows for genome-wide analysis with single-base resolution (Reinders and Paszkowski 2010). Here, we performed the first bisulfite sequencing of any ctenophore by creating a methylome of *M. leidyi* (BioProject ID: PRJNA280770). We sequenced to $\times 38$ coverage of the genome and had an average of $\times 26$ depth of coverage. Our data show that 349,250 or 17.69% of the total CpG sites were methylated, which makes up just 0.22% of the total this genome (Table 1). After filtering for sites with at least a 30% methylation rate this number shifted to approximately 0.09% genome-wide CpG methylation, which is very similar to our findings from ELISA assays (Fig. 3A, B; Table 1). After trimming, 69.9% of the bisulfite-sequence data were mapped to the *Mnemiopsis* genome and covered 1.9 million of the approximately 4.4 million total CpG sites (43% of total CpGs). We also would like to make a technical note with respect to the analysis of the data on methylation. Aside from the scope of the coverage and the rate of bisulfite conversion of unmethylated cytosines to uracils (99.16%), one of the main factors in mapping bisulfite data is the quality of the genome assembly. Since the conversion of bisulfite turns all unmethylated Cs into Ts these data are essentially in 3-base space making it more difficult to map (Reinders and Paszkowski 2010). If the assembly of the genome has imperfections it only complicates



C

Nucleotide	Total Number in Genome
C	29,218,363
A	45,939,243
T	45,971,580
G	29,209,060
N	5,527,301

Fig. 3 Genome-wide rates of contextual methylation are lineage-specific. (**A**) Percentages of genomic methylation across metazoa. Different contexts of methylation, including CG, CHG, and CHH were obtained from bisulfite sequencing. *Mnemiopsis leidyi* (CG = 0.09%, CHG = 0.03%, CHH = 0.11%); *Nematostella vectensis* (CG = 1.44%, CHG = 0.00%, CHH = 0.00%) (Zemach et al. 2010); the data from *Apis mellifera* (CG = 1.65%, CHG = 0.60%, CHH = 2.00%) are an average from Lyko et al. (2010) and Cingolani et al. (2013). The data from *Crassostrea gigas* (CG = 1.95%, CHG = 0.00%, CHH = 0.01%) (Wang et al. 2014); the data from *Homo sapiens* (CG = 4.15%, CHG = 0.35%, CHH = 1.32%) are an average from Lister et al. (2009, 2013) and Li et al. (2010). The pie charts above each species represent the distribution of methylation of CG versus CHG versus CHH in the genome. (**B**) ELISA-based colorimetric assay to validate presence of 5-mC in *Mnemiopsis*, *Beroe*, and *Pleurobrachia*. The level of 5-mC DNA methylation in *Pleurobrachia* (~1.0%) is about 10-fold that of *Mnemiopsis* and *Beroe*. (**C**) The distribution of nucleotides in the genome of *Mnemiopsis* shows a reduced representation of cytosine and guanine nucleotides and a notable amount of N values. (**D**). Methylation in *Mnemiopsis leidyi* in the context of promotor versus gene-body shows that ctenophores have higher promoter methylation (7.44%) than exon or intron methylation (4.59% and 4.95%, respectively). (This figure is available in black and white in print and in color at *Integrative and Comparative Biology online*)

this process. We determined that there are 5,527,301 *N* values (unknown bases) in the genome sequence of *Mnemiopsis* (Fig. 3C), which may affect the mapping. The same issue of *N*-values, especially in intergenic regions, may also affect the reported genome-wide statistics on methylation in other invertebrates. Single nucleotide polymorphisms (SNPs) might also be affecting the accuracy of any methylation mapping, SNPs in a particular gene have also been linked with changes in the patterning and gene expression of 5-mC between different populations of *Apis* (Wallberg et al. 2014).

In *Mnemiopsis*, upstream gene-promoter regions (2 kb) show a higher percentage of methylated CpGs (7.44%) than do gene bodies (4.78%), while there is no difference in percent methylated CpGs between introns (4.95%) or exons (4.59%) (Fig. 3D).

A comparison of the genome-wide statistics of methylation in *Mnemiopsis* to other metazoan bisulfite-sequencing projects is shown in Fig. 3A. *Nematostella* has a genome-wide CpG methylation rate of 1.44% (Zemach et al. 2010). The honey bee, *Apis*, shows CpG methylation percentages ranging from 0.9% to 2.5% (Lyko et al. 2010; Cingolani et al. 2013). The molluscan bivalve *C. gigas* has a value of 1.95% (Wang et al. 2014) and the average genome-wide CpG methylation in humans is 4.15% (Fig. 3A) (Lister et al. 2009). Overall *Mnemiopsis* had lower genome-wide levels of methylation of CpG, CHG, and CHH than those reported from most other metazoans, but has levels comparable to some species of fire ants and other insects (Bonasio et al. 2012).

Our data also demonstrate for the first time that ctenophores have methylation at non-CpG sites, such as CHG and CHH, where the H = T, C, or A. *Mnemiopsis*' percentage of methylation for CHG across the genome is 0.12% (0.03% adjusted) and for CHH is 0.39% (0.11% adjusted) (Table 1,

Table 1 Methylation context in the genome of ctenophore *Mnemiopsis leidyi* (bisulfite sequencing)

Methylation context	Total sites in genome	Total sites called	Total methylated sites	Total methylated sites adjusted	% Methylated	% Methylated adjusted	% of Genome	% of Genome adjusted
CpG	4,405,646	1,974,500	349,250	135,877	17.69	6.88	0.22	0.09
CHG	5,279,032	2,707,653	189,301	40,102	6.99	1.48	0.12	0.03
CHT	6,775,269	2,557,641	201,021	52,718	7.86	2.06	0.13	0.03
CHA	6,927,842	2,452,424	200,112	33,033	8.16	2.04	0.13	0.03
CHC	4,367,411	2,258,129	208,738	71,682	9.24	3.17	0.13	0.05
CHH	18,070,522	7,268,194	609,871	174,327	8.39	2.40	0.39	0.11

Fig 3A). Once again, these numbers are lower than for other metazoans, but *Mnemiopsis* has a distribution of methylation similar to that of *Apis* in that CHH methylation makes up the largest portion of its methylated sites (Fig. 3A).

DNMT-associated proteins

The DNMT1 enzyme has a DMAP binding domain for recruiting DNMT1-associated protein that is conserved in chordates, as well as two BAH domains that facilitate protein–protein interaction and appear to be conserved in all metazoans. DNMT1 is both recruited by, and also recruits proteins from, many complex epigenetic pathways, either via a direct binding interaction or via protein-recognition of DNA-methylation marks (Rose and Klose 2014). For instance, methyl-binding domain (MBDs) proteins recognize methylated but not hemi-methylated CpG sites and recruit histone-modifying enzymes, such as histone deactylase1 (HDAC1), to condense nucleosomes and lead to the repression of genes (Sarraf and Stancheva 2004). The extent to which DNMT1-associated proteins are conserved in non-chordates has not been assessed.

Quantitative transcriptome profiling of *DNMT1* in *Pleurobrachia* demonstrated that this gene was expressed across development, predominantly in early cleavage with high *TET* (demethylation enzyme) expression in the adult combs (Moroz et al. 2014). We reanalyzed and expanded upon this transcriptome profiling by identifying homologs in *Pleurobrachia* for protein-coding genes known to associate with DNMT1 according to the database on STRING protein interaction (Supplementary Material) (Szklarczyk et al. 2011).

Across all of the *Pleurobrachia* developmental transcriptomes, the 4 through 32 cell-stages showed the highest expression levels for *DNMT1* and two associated sequences, *HELLS* and *RUVB1*, but not for the *TRDNMT1* sequence (Fig. 4A). The hierarchical heat map shows a similar pattern in which the levels of expression of the sequences for the DNMT1-associated proteins are also elevated; however, several of the patterns of gene expression appear to be unique to *Pleurobrachia* (Fig. 4B and Supplementary Material Excel Table). Interestingly, no DMAP-binding domain has been identified in any ctenophore DNMT1 genes, and there is no correlation between the transcript expression of the only identified DMAP1-like sequence in *Pleurobrachia* and the identified *Pleurobrachia* DNMT1 gene (Fig. 4B). We also identified a methyl-binding domain in the *Pleurobrachia* genome (PF01429), but the sequence to which it belonged was not identified as a member of the MBD protein family.

One of the most abundant DNMT-interacting proteins expressed during early in ctenophore development is Lymphoid Specific Helicase (HELLS). In other species, the interaction of DNMT1 with HELLS is necessary for *de novo* methylation, maintenance of methylation, and the growth and divisions of cells (Myant et al. 2011). In *Pleurobrachia*, the expression level of HELLS tapers off near the end of development and remains relatively low in the adult tissues that were investigated (Fig. 4A, B). In bilaterians, the HELLS helicase silences expression of stem-cell genes by inducing DNMT1 to methylate selected promoter regions (Xi et al. 2009). In mice, HELLS knockouts had irregular patterns of DNA methylation and had developmental defects (Yu et al. 2014). Through its correlated activity with DNMT, HELLS also activates expression of the cell-cycle-regulating transcription factor E2F3, and HELLS depletion causes down-regulation of E2F target genes (von Eyss et al. 2012). Both E2F and RB1 are required for proper function of E2F, but their co-expression is not detected until the 8-cell stage in *Pleurobrachia*, thereby suggesting HELLS and DNMT1 might be interacting to regulate expression of stem-cell related genes during early cleavage in ctenophores.

In contrast, another helicase, the ATP-dependent Holliday junction DNA helicase (RUVB1), which is most known for its ability to unwind supercoiled

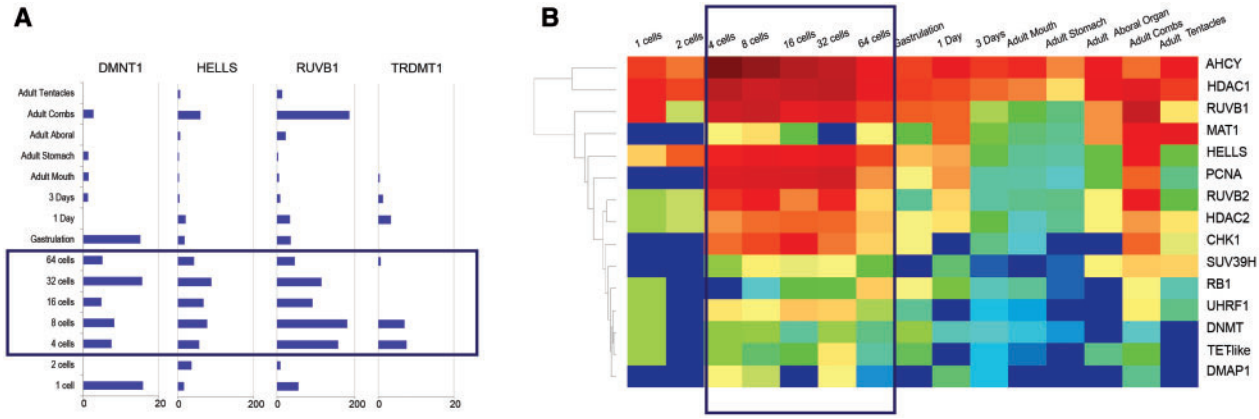


Fig. 4 RNA expression of DNA methyltransferases (DNMT1) and associated helicases in the ctenophore *Pleurobrachia* across development and in adult tissues. Expression is presented as normalized transcripts per million (TPMs). (**A**) The RUVB1 helicase shows several-fold higher expression even in adults' tissue than does the HELLS helicase which recruits DNMT for *de novo* methylation. The presence and relative abundance of this enzyme, especially during early-stage development, suggests a role for DNMT1 in *de novo* methylation in ctenophores. (**B**) Heatmap of expression of all DNA-associated protein transcripts (TPMs) identified in *Pleurobrachia* shows that the most abundant expression of DNMT-associated proteins occurs during the 4-cell to 32-cell stages. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology online*)

DNA and not directly associated with the act of methylation, has several-fold higher expression throughout the development of *Pleurobrachia* and is the most abundant DNMT1-associated protein in most tissues of adults (Fig. 4A, B) (Ahmad and Tuteja 2013). Further discussion of the expression patterning of DNMT1-associated proteins can be found in the Supplementary Materials.

Global 6-mA DNA methylation

In eukaryotes

An additional class of DNA methylation events, which are possibly functionally novel, are being described due to improved capabilities for detection and other methodologies. In prokaryotes, the 5-mC DNA methylation mark is not as common as in eukaryotes. The predominant form of methylation in prokaryotes is the result of a methyl group being added to the sixth position of the purine in adenine (6-mA) (Iyer et al. 2011; Kumar and Rao 2013). However, recent studies convincingly report the presence of 6-mA in three different eukaryotic genomes: the green alga *Chlamydomonas reinhardtii* (Fu et al. 2015), the nematode *C. elegans* (Greer et al. 2015), and the fruitfly *D. melanogaster* (Zhang et al. 2015).

Modifications of both 5-mC and 6-mA DNA have been detected in *Chlamydomonas*, but now with advanced sequencing techniques it has been shown that 6-mA is enriched at gene promoters, particularly at transcription start sites; these covalent DNA modifications do correlate with an increase in transcription activity (Fu et al. 2015). In contrast, 5-mC methylation in *Chlamydomonas* mostly accumulates in gene bodies (Fu et al. 2015). Overall, 6-mA methylation occurs in approximately 0.4 mol% 6 mA/A or 0.1% of the genome (Fu et al. 2015).

Surprisingly, 6-mA DNA methylation has also been detected in *C. elegans* using an extensive array of techniques, including ultra-performance liquid chromatography-tandem mass spectrometer (UHPLC–MS/MS) (Greer et al. 2015) (Supplementary Material). In this nematode, 6-mA DNA methylation is involved in epigenetic transgenerational inheritance (Greer et al. 2015). Overall the levels of 6-mA methylation are very low in wild-type *C. elegans* (0.025% 6-mA/A or 0.0065% of the genome); yet, it increases 10-fold in mutants lacking histone demethylase (Greer et al. 2015).

In *Drosophila*, 6-mA methylation was detected on or near transposons in the early developmental stages (Zhang et al. 2015). As was shown in *C. elegans*, also using UHPLC-MS/MS and immunocytochemistry, detected levels of 6-mA methylation were very low, targeting only 0.07% 6-mA/A or 0.0175% of the genome (Zhang et al. 2015).

The proposed machinery for the 6-mA methylation/demethylation is highly conserved. In *C. elegans*, Greer et al. (2015) proposed the 6-mA methyltransferase is the *DAMT-1* gene (human ortholog: *METTL4*). The demethylase enzymes are members of the alkylation repair 4 homolog (ALKB) dioxygenases family and are designated as NMAD in *C. elegans* (Greer et al. 2015), as a TET-like enzyme in *Drosophila* (Zhang et al. 2015) and as ALKB4 in humans.

The evidence for alternative DNA methylation in ctenophores

We identified the machinery for functional 6-mA methylation in ctenophores (Fig. 5A). Specifically, we conducted quantitative transcriptome profiling for the 6-mA methylase and demethylase genes in the aboral organ of *Mnemiopsis* (Fig. 5B). Here the 6-mA demethylation gene has double the expression levels of the methylase gene (Fig. 5B). Searches of the genome of *Pleurobrachia* and eight other species of ctenophores for homologous sequences indicate a similar gene complement (Moroz et al. 2014; Moroz 2015). Interestingly, ctenophores appear to have an expansion of the demethylation ALKB-like enzymes compared with other basal metazoans. In the genome of *Mnemiopsis*, we identified over 20 ALKB-like genes, and more than 11 ALKB-like genes were found in the genome of *Pleurobrachia*. We refer to these genes as "like" since none has been expressed or characterized.

Next, we experimentally detected the presence of 6-mA DNA methylation in three species of ctenophores, *Mnemiopsis*, *Beroe*, and *Pleurobrachia* at 0.02%, 0.01%, and 0.025% of the genomes, respectively (Fig. 5C). Using a specific antibody to the 6 m adenine nucleotide in an ELISA platform, we show that the genomes of *Pleurobrachia* and *Mnemiopsis* have similar amounts of 6-mA, whereas *Beroe* has lower levels of 6-mA in its genome (Fig. 5C) (Supplementary Materials). Overall, the level of 6-mA methylation in the genomes of ctenophores is approximately four times more than in *C. elegans*, double that of *Drosophila*, but not as high as in the genome of *Chlamydomonas*.

It should be noted that besides 6-mA DNA methylation, 6-mA marks might also occur as RNA methylation events. The predicted enzymes that perform 6-mA RNA methylation/demethylation are also detected in all the ctenophores that were screened. Extensive care was taken to ensure that the detected 6-mA was not due to RNA by treating isolated genomic DNA with RNases. In addition, we also validated the lack of potential RNA contamination in our isolated samples by running gDNA on a RNA-specific tape on a TapeStation (Agilent Technologies). However, without evidence from direct 6-mA-specific sequencing or HPLC analysis, a minor chance of residual RNA cannot be completely excluded. In summary, we have just begun to elucidate the role of 6-mA DNA methylation in ctenophores. More extensive and sensitive measures of 6-mA DNA methylation should be employed together with functional analysis of this novel class of epigenetic regulation in metazoans.

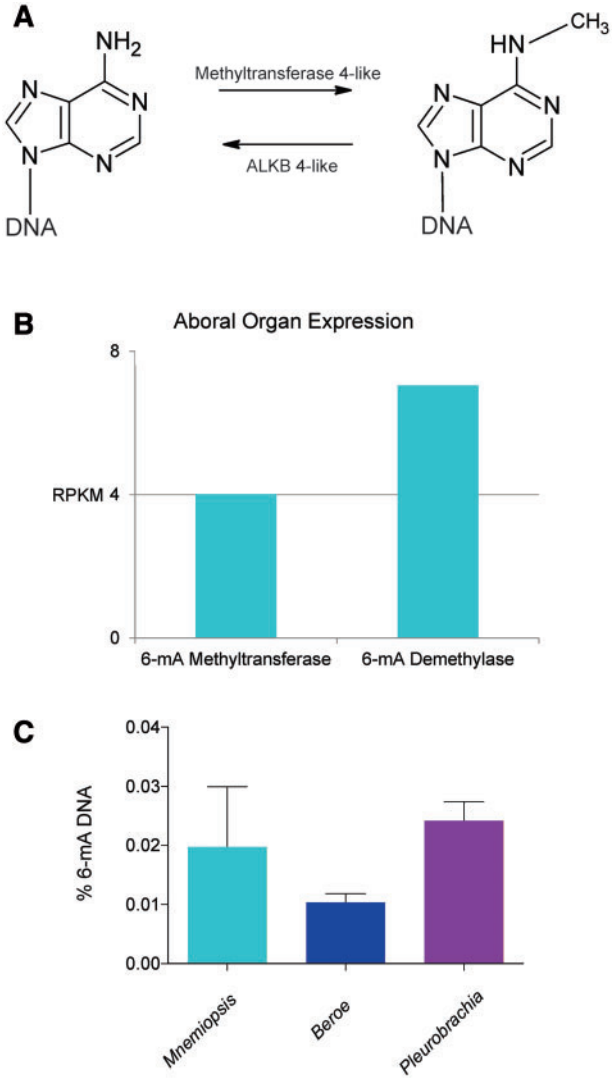


Fig. 5 6-mA DNA methylation in ctenophores. (**A**) Enzymatic mechanisms for 6-mA methylation/demethylation in DNA. Proposed enzymes for 6-mA methylation/demethylation acting on DNA and their resultant products are shown. (**B**) Quantitative expression of the predicted 6-mA methylation/demethylation in the *Mnemiopsis leidyi* aboral organ. The putative demethylase (ALKB 4-like) displays twice the amount of expression as the putative methyltransferase 4-like enzyme in the aboral organ of *Mnemiopsis* (see Supplementary Materials for details). (**C**) ELISA-based colorimetric assays to validate the presence of 6-mA methylation in the genomes of *Mnemiopsis*, *Beroe*, and *Pleurobrachia*. Both *Pleurobrachia* and *Mnemiopsis* genomes contain close to double the amount of 6-mA methylation in their genomes compared with *Beroe* (see Supplementary Material for details). (This figure is available in black and white in print and in color at *Integrative and Comparative Biology online*)

Discussion and future directions

Figure 6 summarizes the current view of the evolution of the DNA methylation machinery in metazoans. High identity of the DNA-methylase domain in DNMT1-like proteins suggests that these *DNMT1*

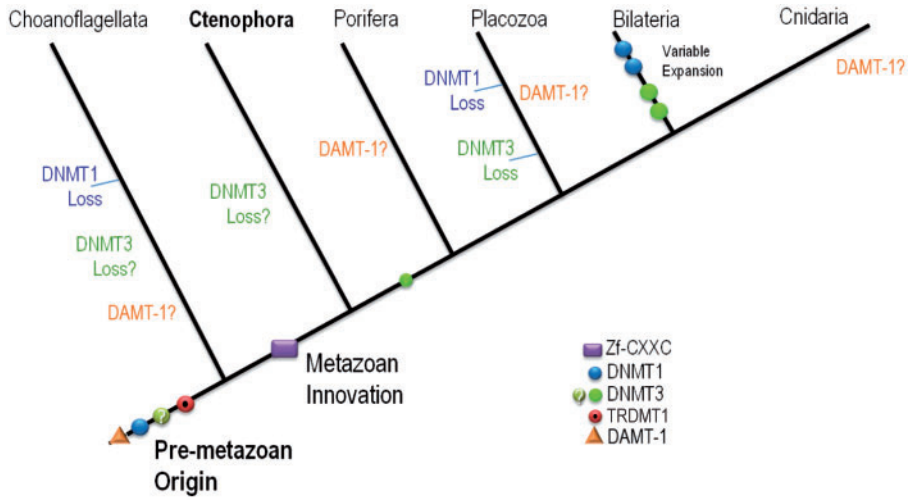


Fig. 6 Evolution of the DNMT families of genes. The canonical DNMT1 originated early in eukaryotic evolution but the zf-CXXC domain is a metazoan-specific innovation. Choanoflagellates and the placozoan Trichoplax adhaerens have both lost DNMT1. TRDMT1 is the only gene conserved in every metazoan lineage, but it actually functions as a tRNA methylase. DNMT3s have putative homologs in prokaryotes, and flowering plants. DNMT3 is absent in choanoflagellates and ctenophores but is present in Porifera, cnidarians, and bilaterians. The 6-mA DNA methyltransferase, DAMT-1, is present in unicellular eukaryotes, and has now been confirmed in ctenophores and bilaterians. Although DAMT-1 is known to be present in prokaryotes and algae suggesting a premetazoan origin, the scope of gene loss and gene gain of DAMT-1 has not been systematically evaluated across metazoans. See text for details. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology online*)

genes shared a common ancestry with prokaryotic *DNMT1*-like genes, but the complex organization of this gene such as the acquisition of the zf-C-X-X-C domain may be a metazoan innovation (Fig. 6). The origins of *DNMT1*-like or *DNMT3*-like genes are not resolved, though it appears that *DNMT1* and *DNMT3* are not derived from ancestral TRDMT1-like genes, which are present in prokaryotes (Jurkowski and Jeltsch 2011). Most likely, the metazoan *DNMT1* and *DNMT3* family of genes independently evolved from ancestral prokaryotic methyltransferases. However, further comparative enzymatic analysis of these families is a necessity for drawing a definitive conclusion.

Most metazoan clades have a DNMT1-like enzyme with the exception of the placozoan *Trichoplax*, suggesting a loss of 5-mC DNA methylation in this lineage. In contrast, the presence of a *DNMT3*-like gene across metazoans is very mosaic. Vertebrates and insects both have undergone expansions in the *DNMT3* gene, with mammals having three *DNMT3* genes. One DNMT3 sequence has been identified in the genomes of *Nematostella* and *Amphimedon*, but there is no DNMT3 in the sequenced ctenophores.

Given the absence of DNMT3s in choanoflagellates, fungi, unicellular eukaryotes, algae, and also in ctenophores, it is hard to discern whether all eukaryotic DNMT3s share a common origin with extensive losses of DNMT3, or whether the DNMT3s of metazoans and flowering plants are a product of convergent evolution (Fig. 6). The origins of DNMT3 enzymes are unclear. There are bacterial proteins containing just a DNMT3-like domain, but the functional role of these proteins has not been assessed (Iyer et al. 2011). Angiosperms (flowering plants) possess a unique domain-rearranged methyltransferase (DRM) type of DNMT3. However, the DRM catalytic methyltranferase domain underwent a circularization permutation and instead of a PWWP domain (Fig 1), DRM proteins have one to three n-terminal ubiquitin domains (Pavlopoulou and Kossida 2007). These DNMT3-like DRM enzymes are found in several sequenced species of angiosperms, but not in the genomes of either sequenced alga (Pavlopoulou and Kossida 2007). DRMs are recruited to perform *de novo* methylation by the presence of siRNAs and associated plant-specific RNA-polymerases (Matzke and Mosher 2014). Previous phylogenetic analyses have shown a strong support for the clustering between plant and metazoan DNMT3 sequences; however, no invertebrate or basal metazoan DNMT3 sequences were used in reconstruction of those genealogies (Pavlopoulou and Kossida 2007; Jurkowski and Jeltsch 2011).

Presence and diversity of a DNMT1-like gene correlates with the ability to methylate cytosines (Fig. 6). The genome-wide level of CpG methylation found in *Mnemiopsis* (0.09%) is comparable to the levels

found in the fire ants *Camponotus floridanus* (0.14–0.16%) and *Harpegnathos saltator* (0.11–0.12%), in which gene methylation was positively correlated with RNA transcript expression in both genomes (Bonasio et al. 2012).

Importantly, DNA methylation can also potentially buffer the evolution of proteins. Sarda et al. (2012) analyzed the context of the evolution of protein sequences and the methylation of gene-bodies in the cnidarian *N. vectensis*, the chordate *Ciona intestinalis*, and the insects *A. mellifera* and *Bombyx mori*. Genes with consistently high levels of gene-body methylation had the lowest rates of evolution of proteins, even across distantly related species (Sarda et al. 2012). A similar study comparing mammals also showed a correlation between high gene-body methylation and low rates of protein evolution; additionally a positive correlation between high promoter methylation and increased protein evolution was found (Chuang and Chiang 2014). The relatively higher promoter region and lower gene-body methylation in *Mnemiopsis* could potentially be correlated with the rate of protein evolution in ctenophores as well.

Future directions

Our initial data on the detection of 6-mA DNA methylation in ctenophores have further suggested a new dimension of epigenetic regulation in metazoans. Components of the 6-mA DNA methylation machinery have now been detected in several species of ctenophores. The respective enzymes are highly conserved across all domains of life. Interestingly, ctenophores appear to have an expansion of the demethylation ALKB-like enzymes.

One possibility is that ctenophores could be utilizing the epigenetic marks of 5-mC and 6-mA DNA methylation similar to *Chlamydomonas*, where 5-mC and 6-mA methylation are used to regulate different regions (i.e., promoters, transcription start sites, exons, or introns) and even different states (active or repressive transcription) of the genome (Fu et al. 2015). Further analysis including UHPLC-MS/MS, sequencing, and immunocytochemistry will be needed to address the functional role of methylation in ctenophores.

Conclusions

We have demonstrated that the genomes of both *Pleurobrachia* and *Mnemiopsis* contain 5-mC DNA methylation, albeit at lower genomic levels compared with other metazoans. Also, for the first time, we performed the quantification of 6-mA DNA methylation in any basal non-bilaterian metazoans. Further exploring the interplay between the 5-mC and 6-mA methylation marks holds the enormous potential to decipher mechanisms of epigenomic regulation not only in ctenophores, but also to expand our understanding of the evolution of DNA methylation in metazoans.

Methods

Detailed methods are provided in the Supplementary Materials. Briefly, alignments of proteins were performed with MUSCLE, trimmed with gblocks, and trees were generated using RAxML 8.0. For ELISA, genomic DNA isolations from whole animals were performed using Qiagen genomic tip protocols according to the manufacturer's recommendations. gDNA was used to perform Epigenetik 5mC (Cat No. P-1034) or 6-mA (Cat No. P-9005) Colorimetric Assays. The antibody used in the 6-mA assay recognizes the nucleotide 6 m-adenine and can be used to assay both RNA and DNA. Extensive care was taken to ensure that the detected 6-mA was not due to RNA contamination by treating the isolated gDNA with RNases. We validated the absence of RNA contamination in the isolated gDNA by analyzing the gDNA on a RNA-specific tape using the TapeStation (Agilent Technologies). For bisulfite sequencing: Genomic DNA from one *M. leidyi* was isolated, using an Qiagen DNeasy kit. The EZ methyldirect bisulfite conversion kit was used and methylation libraries for sequencing were prepared with EpiGnome Methyl-seq protocol. Methylation data were analyzed using the MOABS pipeline (Sun et al. 2014).

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Supplementary data

Supplementary data available at *ICB* online.

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