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Beyond transcriptional silencing: Is methylcytosine a widely conserved eukaryotic DNA elimination mechanism?

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Abstract

Methylation of cytosine DNA residues is a well-studied epigenetic modification with important roles in formation of heterochromatic regions of the genome, and also in tissue-specific repression of transcription. However, we recently found that the ciliate *Oxytricha* uses methylcytosine in a novel DNA elimination pathway important for programmed genome restructuring. Remarkably, mounting evidence suggests that methylcytosine can play a dual role in ciliates, repressing gene expression during some life-stages and directing DNA elimination in others. In this essay, I describe these recent advances in the DNA methylation field and discuss whether this unexpected novel role for methylcytosine in DNA elimination might be more widely conserved in eukaryotic biology, particularly in apoptotic pathways.

Keywords

apoptosis; epigenetics; genome rearrangement; methylcytosine; *Oxytricha*

Introduction

Cytosine methylation is an ancient epigenetic modification that plays well-known biological roles in the bacterial antiviral response [1], eukaryotic genome stability [2], and transcription repression [2]. The transcriptional repressive function of methylcytosine has been best characterized at CpG motif clusters (known as “CpG islands”) that are frequently found in gene promoter regions [3]. Methylated cytosine also suppresses transposon mobilization in animals and plants [4, 5]. However, comparison of methylcytosine distributions in a variety of plants, animals, and fungi demonstrates that methylation of gene bodies (i.e. coding sequences) is the most widely conserved pattern, where its functional role is unknown but potentially linked to RNA splicing [6, 7]. The complexity of cytosine methylation patterns is a testament to its ancient origin and subsequent lineage-specific evolutionary adaptations; indeed, in some cases (including most common model organisms), methylcytosine has been lost altogether [8], presumably because of its inherent mutagenicity, as it spontaneously

deaminates into thymine [9]. As a deeply diverging eukaryote [10], the ciliate *Oxytricha trifallax* offers important insight into the history of methylcytosine.

A defining feature of ciliates is nuclear dimorphism: in every sexually viable cell, these unicellular eukaryotes harbor two types of nuclei (Fig. 1A), each enclosing a different genome [11]. The physically larger, polyploid macro-nucleus, or MAC (Fig. 1A) produces all known mRNA necessary for vegetative (asexual) cell growth and division (see Box 1 for glossary). The physically smaller, diploid micronucleus or MIC (Fig. 1A) is vegetatively silenced, and serves as a germline, transmitting DNA to the next generation. Under conditions of low food, cells of compatible mating type conjugate, undergo meiosis and fertilization, and initiate a remarkable genomic developmental process (Fig. 1B–E) in which a new (zygotic) MAC is produced, containing the genomic contributions of both parental cells [11]. Concurrently, the parental MAC is destroyed along with the entirety of its genomic contents (Fig. 1B–F) [11] in a process known as programmed nuclear death (PND), a phenomenon combining apoptosis [12] and autophagy [13] to accomplish DNA fragmentation and organelle resorption.

Box 1

GLOSSARY

MIC	Micronucleus, contains the germline genome of <i>Oxytricha</i> , is transcriptionally silent, repeat-rich, and contains scrambled genes.
MAC	Macronucleus, contains the somatic genome of <i>Oxytricha</i> , is actively transcribed during cell growth and asexual division, and contains thousands of short, polyploid chromosomes usually bearing single genes.
MDS	Macronuclear-destined sequence, DNA regions in the micronucleus that ultimately are retained during formation of a new macronucleus.
IES	Internal eliminated sequence, spacer DNA inserted into genes, and must be excised during macronuclear development.
TBE	Telomere bearing element, a class of transposable elements definitive of the <i>Oxytricha fallax</i> sibling species complex (including <i>O. trifallax</i>) and containing terminal telomeres internal to the host MIC chromosome.
ORF	Open reading frame, is the protein-coding region on a chromosome or mRNA.

PND	Programmed nuclear death, analogous to programmed cell death (apoptosis) in multicellular organisms, but involving the destruction of a nucleus, not a whole cell.
TET	An enzyme of the ten eleven translocated dioxygenase family, responsible for producing hydroxymethylcytosine (and other oxidized derivatives) from methylcytosine.
H3K9me3	Histone 3 lysine 9 trimethylation, a repressive chromatin mark.
H3K27me3	Histone 3 lysine 27 trimethylation, a repressive chromatin mark.

The process of zygotic nuclear development is elaborate in *Oxytricha*, involving the cutting and exact splicing together of short DNA segments known as macronuclear destined sequences (MDSs), and elimination of hundreds of thousands of gene-interrupting internal eliminated sequences (IESs) that cripple most coding sequences in the MIC [14]. In total, over 80% of MIC genomic DNA is destroyed [15], and the remaining DNA, comprising relatively short genic fragments, is stitched together to produce over 16,000 short, polyploid chromosomes, averaging 3.2 kb in length, capped with short telomeres, and most containing only a single open reading frame [16] (Fig. 1H). In addition to the IESs, eliminated DNA includes repetitive elements such as the telomere bearing element (TBE) transposons [17] and satellite repeats [18] (Fig. 1H), as well as intergenic sequence.

A dual role for heterochromatic histone modifications in *Tetrahymena*

The analysis of heterochromatic, repressive DNA modifications in *Tetrahymena* revealed a surprising dichotomy: they can be both dynamic and static. Instead of delineating stable domains of transcriptional repression as in most eukaryotes, histone 3 lysine 9 trimethylation (H3K9me3) is deposited on DNA destined for elimination during genome rearrangements [19–21] (Table 1), so it essentially removes itself from the vegetative genome, becoming virtually undetectable in the exconjugant cells (i.e. post-conjugation) [20, 22]. However, another repressive histone modification, trimethylated histone 3 lysine 27 (H3K27me3), plays a dual role: it participates in elimination of DNA and is also found in the vegetative cell macronucleus and micronucleus [20] (Table 1), where presumably it helps maintain regions of transcriptionally silent heterochromatin. During genome rearrangements, both H3K9me3 and H3K27me3 are dynamically targeted to IES elements [19–21] and are vital for proper IES excision [20, 21] (Table 1). A remarkable small RNA pathway targets histone methylation to IESs during rearrangements (reviewed in [23]). Two chromodomain-containing proteins, Pdd1p and Pdd3p, are expressed during genome rearrangements [24, 25], associate with IESs during their elimination, and directly bind H3K9me3 [20] (Table 1). Pdd1p has also been shown to bind H3K27me3 [20] and, significantly, is capable of inducing DNA elimination of heterologous sequences when it is artificially tethered directly to the DNA [19], suggesting that it plays an important effector role in DNA elimination processes.

Taken together, these data demonstrate dual functional roles for heterochromatic histone modifications in *Tetrahymena*: transcriptional silencing of genes during vegetative growth (H3K27me3), and elimination of DNA during genome rearrangements (both H3K27me3 and H3K9me3) [20]. The developmental expression of chromo-domain proteins, which specifically recognize methylated histone tails, may re-code these heterochromatic marks to specify DNA elimination instead of transcriptional silencing. Accordingly, Pdd1p localizes to both parental MAC (during PND) and zygotic IESs (Table 1) [24, 26, 27], though genetic knockouts have conclusively demonstrated only its role in IES excision [26, 28], leaving open the possibility that another, currently unknown chromodomain protein may mediate DNA elimination in PND.

Echoes of these pathways resonate across vast stretches of evolutionary distance, to the metazoan and fungal branch of the eukaryotic phylogenetic tree. For example, in fission yeast, formation of functional centromeres involves recognition of centromeric repeats by small RNAs that direct H3K9 methylation; as in *Tetrahymena*, chromodomain proteins recognize these histone modifications [29]. In the *Drosophila* germline, transposable elements are silenced by H3K9 methylation that is targeted by small RNAs [30] and –not surprisingly – a chromodomain protein has been linked to the silencing process [31]. In mouse testes, the silencing of transposons occurs by de novo cytosine methylation [32], and likewise in the ciliates *Oxytricha* and *Stylonychia* transposons are first methylated and then eliminated during genome rearrangements [33, 34] (Fig. 1, Table 1). In ciliates, therefore, deeply conserved eukaryotic heterochromatin pathways have been exaggerated into the ultimate form of genetic silencing: destruction of the marked DNA.

Dueling DNA methylation models in *Oxytricha* and *Stylonychia*

DNA methylation was initially thought to have been lost in ciliates: early investigations found that methylcytosine was undetectable in *Tetrahymena* [35], *Paramecium* [36], *Oxytricha* [37], and *Stylonychia* [38]. However, reports of methylcytosine's demise in ciliates were greatly exaggerated, as a subsequent study reported methylcytosine in the macronucleus of *Blepharisma* [39], and another found a role for methylcytosine during encystment of *Colpoda* [40]. Even the reported absence of methylcytosine in *Stylonychia* proved short-lived, as a study showed evidence of de novo methylation of cytosine on a germline repetitive element during genome rearrangements [34]. Our discovery of methylcytosine in *Oxytricha* [33] is therefore consistent with the tradition of resurrecting methylcytosine from its premature dismissal in various ciliate lineages. In *Oxytricha*, we reported the rearrangement-specific de novo deposition of methylcytosine (and recently rediscovered hydroxymethylcytosine [41]) on repetitive elements, the parental macronuclear genome, and in errors of genome splicing [33] (Fig. 1F and H). More recently, we have discovered that single-copy IESs that are excised as circular forms are also methylated, and that unmethylated versions of these IES circles were undetectable (two loci tested, J.R. Bracht, L.F. Landweber, unpublished work). The observation that every methylated DNA element identified to date is also destined for elimination lends support to a model in which methylcytosine (and possibly hydroxymethylcytosine) acts as a specific marker for DNA loss [33]. Confirming this hypothesis, we showed that inhibition of methyltransferases caused the retention of repetitive elements in the zygotic genome [33] (Table 1). Note also

that the inhibition of DNA methylation effects a *functional nuclear shift* –the retention of repetitive elements in the MAC genome, where they are ordinarily not found (Table 1).

In what follows, I will at times refer to the methylation/hydroxymethylation pathway as the methylation pathway, without making specific assumptions about the oxidation status of the modified cytosine. This convention does not just simplify the discourse:

hydroxymethylcytosine is produced through oxidation of pre-existing methylcytosine by the ten eleven translocated (TET) family of dioxygenases (reviewed in [42]), so we may infer the previous existence of methylcytosine wherever hydroxymethylcytosine is present.

Contrary to initial reports, methyl-cytosine may be present in vegetative *Stylonychia* and *Paramecium* cells, though at levels below the experimental detection limit. In a recent *Stylonychia* study, cytosine methylation was detected via bisulfite sequencing the promoter region of two genes whose expression is repressed during vegetative growth but then rapidly induced during the genome rearrangement process [43] (Table 1). The induction of these genes correlates with loss of the methylcytosine from their promoters [43], though it would be interesting to test whether methyltransferase-inhibiting drugs such as azacitidine or decitabine re-induce vegetative expression of these genes (Table 1). It is important to note that no delineation in this study between hydroxymethylcytosine and methylcytosine was performed, and bisulfite sequencing techniques cannot distinguish the two modifications [44]. In contrast to our *Oxytricha* study, these authors found that the methylation was not induced de novo –it was stably maintained in the MIC precursor DNA of vegetative cells, then passively inherited in the MAC through the process of nuclear development [43] (Table 1). The evidence for methylcytosine in *Paramecium* is very indirect. Two studies found that azacitidine and decitabine affect cellular recovery after micro-nuclear ablation [45, 46], suggesting that methylcytosine must be present even though it was not directly detected [36].

The recently proposed models of methylcytosine function in *Oxytricha* [33] and *Stylonychia* [43] are near inverses of each other (Table 1). While large-scale evolutionary innovation in methylation pathways between these species cannot be ruled out, it is possible that the conflicting models are in fact simply describing two very different modalities for methylated cytosine in different parts of the ciliate lifecycle – an idea that finds precedent in the dual functions ascribed to histone methylation in *Tetrahymena* (Table 1). Here, I outline a proposed reconciliation of the *Oxytricha* and *Stylonychia* models, and in the final segment I discuss potential conservation of methylcytosine pathways beyond ciliates.

We may account for the apparent lack of methylcytosine in analysis of bulk DNA from vegetative *Stylonychia* [38] and *Oxytricha* [37] by its proposed extremely low overall level in the genome. This would explain the lack of detection in early reports; in our *Oxytricha* study we used ultra high-sensitivity mass spectrometry methods and we observed a small amount of methylcytosine signal in vegetative samples, though we could not rule out contamination from *Chlamydomonas reinhardtii*, the algal food we use in *Oxytricha* culture [33].

However, a specificity problem arises if we suppose that the vegetative *Oxytricha* macronucleus contains low levels of methylcytosine: does it signify transcriptional silencing or elimination? The previously described dual modality of histone methylation marks in *Tetrahymena* provides helpful insight: Pdd1p is a methylated histone-binding protein specifically expressed during genome rearrangements [24], whereupon it recognizes the modified histones [19, 20] and directs DNA elimination of marked sequences [19–21] (Table 1). Thus, in *Oxytricha* the expression of a specific DNA methyl-binding protein (or set of proteins) could quickly re-program methylcytosine – which originally mediated transcriptional repression – to a new functional role, directing DNA elimination. Alternatively, hydroxymethylcytosine is specific to *Oxytricha* genome rearrangements [33], and thus could function as the elimination signal in contrast to methylcytosine, which may function in transcriptional repression in vegetative cells. The most straightforward test of this model would be to inhibit oxidation of methylcytosine by inactivating TET dioxygenase enzymes [41, 42] during genome rearrangements in *Oxytricha*, assaying for elimination defects similar to those observed upon inhibition of methyltransferases [33] (Table 1). While one TET dioxygenase protein is detectable in the *Oxytricha* MAC genome, no cytosine methyltransferase family protein [8] can be detected in either the MIC or MAC genome assemblies (not shown), suggesting that it may be extremely divergent from all known methyltransferase proteins, or reside in difficult-to-assemble genomic regions. We also cannot exclude the possibility that cytosine methylation in *Oxytricha* may occur through a novel pathway. It is worth noting that the *Stylonychia* MAC genome assembly also lacks any known cytosine methyltransferase (E. Swart, personal communication).

In *Stylonychia*, a Pdd1p homolog and chromodomain-containing protein has been identified: Spdd1p [47]. This protein localizes to both zygotic and parental nuclei during rearrangements and elimination, consistent with the behavior of Pdd1p in *Tetrahymena*. Immunofluorescence analysis demonstrated nuclear co-localization of Spdd1p with repressive histone methylation marks during genome rearrangements [48], though the genome-wide analysis of histone modifications in the developing *Stylonychia* genome has not been performed. Given the reports of DNA methylation in this organism, it is tempting to speculate that perhaps in most ciliates both histone and DNA methylation are coordinated to eliminate DNA, and that chromodomain proteins may constitute a conserved downstream mechanism for carrying out the job of DNA destruction.

Are both functional roles for methylcytosine conserved more widely in eukaryotes?

Given the conservation of methylation-silencing functions in eukaryotes, it is natural to investigate whether methyl-cytosine might be playing other conserved, novel roles in manipulating DNA. It was recently shown that homologous recombination (HR)-mediated integration of a transgene in human cells induces localized DNA methylation alterations at the integration site [49]. A molecular link between DNA methylation and repair has been identified: a small, acidic protein known as growth arrest and DNA damage inducible 45 alpha (GADD45α) is activated by both Breast Cancer 1 (BRCA1) and p53 upon the induction of DNA damage [50, 51]. In turn, GADD45α directly binds DNA

methyltransferase 1 (Dnmt1) at its catalytic site, directly inhibiting its methylase activity [52].

Additional evidence that methylation may play important roles in DNA repair comes from in vitro protein pulldown experiments using cytosine, methylcytosine, and oxidized methylcytosine capture probes and mouse nuclear extracts [53]. Intriguingly, many of the immunoprecipitated proteins are components of DNA repair pathways and specifically recognize oxidized forms of methylcytosine such as hydroxymethylcytosine [41] and formylcytosine [54]. These data may be significant, given our finding of hydroxymethylcytosine in *Oxytricha* during genome rearrangements [33], and may point to deeper conservation of DNA methylation pathways in DNA repair.

The elimination of the parental macronucleus in ciliates occurs by a process related to apoptosis [12], combined with autophagy [13, 55], both of which are widely conserved eukaryotic pathways. Strong molecular evidence supports a role for DNA methylation in apoptotic pathways in cancer (for review, see [56]): DNA methylation of pro-apoptotic gene promoters silences them, thereby promoting cell survival and replication. For example, the pro-apoptotic factor Death-Associated Protein Kinase (DAPK) is silenced in certain cancerous B-cell, bladder, and kidney cancer cell lineages by promoter methylation; expression can be restored upon incubation with azacitidine [57].

A more direct role for DNA methylation in directing elimination (rather than transcriptional repression) of sequences from metazoa is both more provocative and more challenging to study. However, recently it was reported that overexpression of DNA methyltransferase 3A (DNMT3A) in mouse motor neurons induces apoptosis, both in vitro and in vivo; more remarkably, inhibition of methyltransferases blocks programmed cell death when induced by DNA damaging agents [58]. The authors also demonstrated significant accumulation of methylcytosine in human motor neurons undergoing neuro-degeneration due to amyotrophic lateral sclerosis (ALS, or Lou Gehrig's disease) [58]. A particular challenge of these studies is that the methylated (or hydroxymethylated) DNA may exist only transiently before it is lost through cell death. Furthermore, in a multicellular organism, apoptosis may only occur in a small number of cells at a specific point in development, making the relevant methylation events quite challenging to capture. This underscores the value of the ciliate model in which millions of cells can be simultaneously induced to undergo genome rearrangements and PND. It will be very interesting to see whether additional model systems for apoptosis will corroborate the striking findings in mouse motor neurodegeneration [58].

To date, ciliates have illuminated two very different DNA methylation pathways: formation of repressive heterochromatin and DNA elimination. It will be interesting to learn the mechanisms by which cells manage to extract two different molecular functions from a single DNA methyl mark, whether through distinct cell-cycle specific "reader" proteins or alternative downstream oxidation steps (which may also be recognized by distinct reader proteins). We may be sure of one thing: ciliates will lead the way in these exciting epigenetic explorations of the mechanisms of methylcytosine in silencing, elimination, and beyond.

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Figure 1.

Oxytricha nuclear structure and overview of genome rearrangement process. **A:** Vegetative (asexual) *Oxytricha* cell containing two nuclei: a germline MICronucleus and a somatic MACronucleus. **B:** After cell mating, a fertilized zygotic micronucleus (∞) is formed. The parental MAC ($\emptyset$) is destroyed by programmed nuclear death (PND) (panels B–E). **C:** The zygotic micronucleus divides, and one becomes the zygotic MAC (v), undergoing radical genome restructuring en route to becoming a new somatic nucleus. Meanwhile, the zygotic MIC (∞) remains as a new germline nucleus (without rearrangement). **D:** As differentiation progresses the zygotic macronucleus (v) enlarges while the parental macronucleus ($\emptyset$) is progressively degraded and lost. **E:** The progeny cell re-enters vegetative growth. **F:** Overview of the molecular events occurring in parental MAC ($\emptyset$) during the genome rearrangement process. For simplicity, only two chromosomes, bearing different open reading frames, ORF1 and ORF2, are shown; there are 16,000 tiny chromosomes in the MAC genome [16]. The chromosomes are constructed of precisely joined MDSs (green arrows) and are capped by short telomeres (gray boxes). During the genome rearrangement process, these tiny chromosomes are degraded (hatched elements in lower panel F), and are also methylated (five-pointed stars). **G:** Overview of the chromosomal structure of a representative zygotic MIC (∞) chromosome segment. The MIC chromosomes (black line) are long and repeat-rich, with telomere bearing element (TBE) transposons shown in orange, and 170 bp satellite repeats in blue; only a portion of a single chromosome is shown. As in F, MDSs are indicated by green arrows; one is scrambled in orientation (MDS2 is inverted) and they are separated by internal eliminated sequences (IESs). **H:** Overview of the molecular events occurring in zygotic MAC (v) during genome rearrangements. Repetitive

elements (shown are TBE transposons, orange, and 170 bp satellites, blue) are excised and eliminated (hatched elements); these sequence elements are methylated (five-pointed stars). In addition, circular IESs and splicing errors are eliminated (hatching) and methylated (stars). The methylation status of intergenic DNA has not been tested. MDSs are excised and re-joined to produce coding potential (ORF, open reading frame, indicated as red arrow), often requiring unscrambling of orientation or order, and short telomeres (gray boxes) are added de novo to their termini.

Table 1

Overview and summary of methylation modifications of a gene promoter in human DNA, *Tetrahymena* histone H3, *Oxytricha* DNA, and *Stylonychia* DNA. Also shown are the functional results of inhibiting methylation (last column). C, cytosine; mC, methylcytosine; hmC, hydroxymethylcytosine; MAC, macronucleus; MIC, micronucleus; Ø, parental macronucleus; v, zygotic macronucleus. Other symbols and abbreviations as indicated in legend in title of “nuclear dynamics” column.

organism	methylation function	nucleus	nuclear dynamics	methylation inhibition
<i>Homo sapiens</i> Law & Jacobson [2]	silencing	only one nucleus present	repressed expression	expression restored
<i>Tetrahymena thermophila</i> Madireddi et al. [24] Taverna et al. [19] Liu et al. [21] Liu et al. [20] Briggs et al. [22]	silencing and elimination	vegetative MAC vegetative MIC zygotic MAC parental MAC
<i>Oxytricha trifallax</i> Bracht et al. [33]	elimination	zygotic MAC parental MAC
<i>Stylonychia lemnae</i> Bulic et al. [43] Juraneck et al. [34]	silencing and elimination	veg MIC veg MAC zygotic MAC