Epigenetic Dynamics in Chlamydomonas: Unveiling New Frontiers in Microalgal Research

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Abstract:

Microalgae exhibit significant diversity and ecological importance, acting as primary producers in water ecosystems and contributing to the biosynthesis of secondary metabolites and other high added-value molecules. Their metabolic flexibility and phenotypic plasticity reflect sophisticated gene expression regulation, largely influenced by epigenetic mechanisms. This review explores the current understanding of these mechanisms in *Chlamydomonas reinhardtii*, focusing on DNA methylation, histone modifications, and non-coding RNAs. Specifically, it examines the roles of 5-methylcytosine (5mC), N6-methyladenine (6mA) and Histone PTMs in gene expression, genome stability, and environmental adaptation. Despite its lower prevalence, 5mC in Chlamydomonas is strategically distributed, affecting gene silencing and transposable elements. In contrast, 6mA, predominantly found around transcription start sites, influences nucleosome positioning and gene transcription. Histone PTMs provide unique landscapes compared to other green organisms. This review also considers the evolutionary implications of these epigenetic marks, proposing that they are essential for environmental adaptation and survival. Further research is needed to elucidate the interplay between these marks and their broader environmental and biotechnological roles.

1. What are microalgae, and why are they an enigma in the realm of epigenetics?

Microalgae represent an exceptionally diverse array of photosynthetic, microscopic organisms, predominantly eukaryotic (including prokaryotic cyanobacteria), covering more than eleven different taxonomic divisions and exhibiting ubiquitous distribution across the biosphere [1], [2].

Their ecological significance is underscored by their role as primary producers, constituting a significant portion of the trophic chain [3], and their involvement in the biosynthesis of numerous secondary metabolites intricately linked to the ecological and chemical dynamics of aquatic ecosystems [4], together with their use in industry. For instance, within the Chlorophyceae class, multiple distinct species of predominantly unicellular algae have been isolated and identified [5]. Many of these species are the focus of research for their diverse biotechnological applications, including biofuel production [6], [7], the extraction of carotenoids [8], [9], and the discovery of novel antibacterial compounds [10]. Moreover, microalgae are investigated for their potential in bioremediation efforts targeting emerging contaminants [11], landfill leachates [12], and the development of innovative wastewater treatment strategies [13]. Simultaneously, they offer promising prospects for integration into industrial systems, serving as agents for carbon sequestration to enhance sustainability [14].

This remarkable acclimation capacity of microalgae, which is the result of their substantial metabolic flexibility and phenotypic plasticity, reflects an underlying ability to regulate gene expression. This regulation is closely related to their epigenetic mechanisms developed in response to various environmental conditions and abiotic stresses [15]. Kronholm et al. (2017) [16] prove how this epigenetic variation can contribute in adaption to different environments: salt stress, phosphate starvation and

high CO2. They changed the amount of epigenetic variation generated or transmitted for two hundred asexual generations and found that reducing the amount of epigenetic variation available can reduce adaption in environments. Given their vast genetic diversity, microalgae exhibit a wide array of epigenetic strategies that have emerged directly from their long-term coevolution in multiple ecotypes. Therefore, a deeper understanding of the regulatory expression mechanisms in microalgae would help to deeper understand the evolution of the chromatin regulation.

Epigenetics can be defined as the mechanisms that regulate gene expression without altering the DNA sequence. These diverse mechanisms are key players in cell growth and development as well as in the response of environmental stimuli. [15], [17], [18], [19]. Epigenome is primarily regulated through the DNA methylation, the chromatin remodeling associated with post-translational modifications (PTMs) of histones, and the small non-coding RNAs (ncRNAs) [15], [20].

Among microalgae, [21]*Chlamydomonas reinhardtii*, the most representative species of this genus, has a ~120 Mb haploid nuclear genome, with an average of 64.1% GC content [22], comprised of 17 chromosomes, a ~205 kbp circular chloroplast genome and a ~15.8 kbp linear mitochondrial genome, exclusively inherited from the parental *plus* mating type (mt+) and the *minus* mating type (mt-) respectively during sexual recombination [21], [23].

This review focuses into the latest research on epigenetic mechanisms in *C. reinhardtii*. Additionally, it examines the similarities and divergences with other green organisms, aiming to address a critical question: Are these mechanisms a highly conserved inherited trait, potentially residual from ancestral lineages, or have they emerged as a convergent coevolutionary adaptation essential for survival under environmental pressures?

2. DNA-Methylation

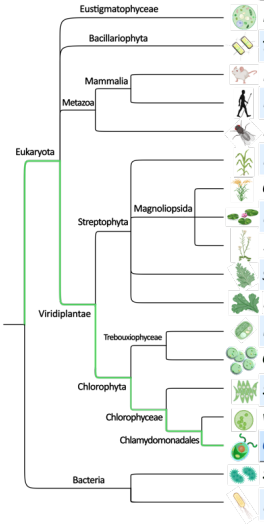
DNA methylation patterns have been estimated to be about five orders of magnitude higher than genetic mutations rates. The evidence for transmission of epigenetic variation opens the question of how epigenetics affects evolutionary adaptation [16]. In *C. reinhardtii* the most abundant and common studied covalent nucleotide modifications of DNA are C^5-methylcytosine (5mC) and N^6-methyladenine (6mA). While 5mC remains the most extensively studied epigenetic mark, 6mA represents the primary form of DNA methylation in this microalgae

2.1 5-methylcytosine

This mark results in the addition of a methyl group ($CH_3\cdot$) at 5th position of the cytosine ring. This is the most prevalent and studied epigenetic mark in eukaryotes, present in almost all species, and has a key role in cell fate, development, and growth. It is involved in gene expression control, gene imprinting, genome stability and transposable elements (TEs) silencing, among other biological processes. In mammals, 5mC is mainly prevalent in CpG islands, and acts as gene transcriptional suppression. In plants is present in CG, CHG and CHH (H: any nucleotide except G), and acts as transposable elements and gene repression, but also maintains constitutive expression when present in gene bodies. As in plants, microalgae 5mC occurs in three different contexts: CpG, CHG, and CHH.

The presence of 5mC in *Chlamydomonas* nuclear genome is substantially lower (~1.3% CG, ~0.3% CHG and ~0.4 % CHH methylation levels; H = A, C or T) [16] than those that has been measured in plants such as *Arabidopsis thaliana* (~22.3% CG, ~5.92% CHG and ~1.51% CHH methylation) , *Oryza sativa* (~59.4% CG, ~20.7% CHG and ~2.18% CHH methylation) [24] or in some microalgae species such as *Picochlorum*

soloecismus (~12.1% CG, 0.8% CHG and 0.9% CHH methylation levels) (Figure 1) [25]. Other green algae such as *Volvox carteri* (~2.6% CG, ~0.08% CHG and ~0.08% CHH methylation context) [26] or *Scenedesmus quadriacuda* (~2.5 % of global 5mC) [27] are more similar to *Chlamydomonas* [25]. Despite its low abundance in this species, its distribution is also quite unique, as it is more selectively concentrated in exons, repetitive regions and long interspersed nuclear elements (LINEs) [28]. Intriguinly, some regions with reduced protein-coding rates were highly hypermethylated [29]. The recent *de novo* genomic assembly of the *C. reinhardtii* CC-5816 strain, showed an average CG enrichment of approximately 23.55% across all centromeres [30], which is consistent with the previous identification of its centromeres as a highly conserved LINE transposable element among different *Chlamydomonas* species such as *C. incerta* and *C. schloesseri* [31]. This suggests 5mC is not a residual evolutionary mark in this species, being involved effectively in TE and gene expression silencing.



	CG	CHG	CHH	Ref	6mA/A	Ref
Nannochloropsis oceanica	n.d.	n.d.	n.d.	[41]	0.1%	[42]
Thalassiosira pseudonana	2.57%	0.13%	0.12%	[36]	#	-
Mus musculus	74.2%	0.30%	0.29%	[28]	0.0005%-n.d.	[43]
Homo sapiens	>80%	n.d.	n.d.	[32]	0.051%-n.d.	[43]
Drosophila melanogaster	0.12%	0.11%	0.11%	[26]	0.204-0.001	[43]
Physcomitrium patens	29.5%	29.7%	23.2%	[26]	#	-
Oryza sativa	59.4%	20.7%	2.18%	[28]	<1%	[44]
Nelumbo nucifera	47.67%	37,56%	13.99%	[38]	1.50-1.19%	[37]
Arabidopsis thaliana	22.3%	5.92%	1.51%	[28]	0.138-0.05%	[43], [45]
Selaginella moellendorffii	0.11%	0.08%	0.08%	[26]	#	-
Marchantia polymorpha	46.40%	28.82%	11.16%	[35]	#	-
Picochlorum soloecismus	12.1%	0.8%	0.9%	[25]	#	-
Chlorella vulgaris	80.5%	2.2%	0.25%	[26]	0.59%	[39]
Scenedesmus acutus	76.9%	2.6%	1.6%	[34]	#	-
Volvox carteri	2.6%	0.08%	0.08%	[26]	0.3%	[46]
Chlamydomonas reinhardtii	1.3%	0.3%	0.4%	[16]	0.4%	[47]
Synechocystis sp.	C^{m5}GATCG:	80-85%	GC^{m4}CC: 90%	[40]	0,001%	[40]
Escherichia coli	CCWGG:	0.75%		[33]	1.5-2%	[33], [48]

Figure 1. DNA methylation across different Eukaryotes and Prokaryotes species. n.d.: non detected; #: data not available.

Box 1. Role of 5mC in the life cycleand in the inheritance of chloroplast DNA (cpDNA) in *Chlamydomonas*.

Several changes in the methylome are observed during *C. reinhardtii* biphasic life cycle. This raises questions about the cellular mechanisms that govern the transition between mitotic and meiotic division. Vegetative cells can remain in mitotic division indefinitely under optimal environmental conditions. In contrast, sexual reproduction is triggered as a survival response to various abiotic stresses, such as nitrogen starvation or light fluctuations [49], with zygospores exhibiting greater resistance. It is likely that other uncharacterized parameters, such as ROS or heat stress, also trigger this response, similar to what is observed in *Volvox* [50], [51]. Whether methylation involved in *C. reinhardtii* sexual behavior is a direct consequence of these environmental effects remains unclear. The distribution of 5mC exhibits dynamic changes in the *C. reinhardtii* chloroplast genome (cpDNA) throughout its life cycle. This methylation mark shows differential increases, reaching approximately 10% in mt+ gametes, 3% in mt- gametes, and up to 54% of CG sites during diploid zygote development [24]. While CHG and CHH methylation levels increase proportionally to average values of around 48% and 39%, respectively, they decrease much more sharply than CG methylation levels after germination [24].

A novel chloroplast-resident DNA methyltransferase, CrMT1, with nonselective cytosine methylation activity, was localized in the chloroplasts of *C. reinhardtii*

[52]. Transgenic insertion of the gene encoding this DMT1 into mt- cells induced a higher percentage (~23%) of biparental cpDNA inheritance, suggesting a methylation-restriction mechanism [53]. This theory posits that 5mC methylation prevents the active degradation of mt+ parental cpDNA by specific endonucleases that would otherwise degrade mt- cpDNA.

Further investigation by López et al. (2015) [24] determined that a locus on chromosome 6, near the MT locus, contains DMT1a in the chloroplast and DMT1b in the mitochondria. Mt- cells show lower 5mC content in cpDNA, lower expression of these enzymes, and a single-base insertion in exon 10 of the DMT1 locus causing a premature stop codon before the methylase domains. Recent research by Joe et al. (2022) [54] identified OTU2p (OTUBAIN PROTEIN 2), encoded in the MT+ locus, as the primary mechanism protecting cpDNA in mt+ cells from degradation since zygotic formation, suggesting that uniparental chloroplast inheritance might be mediated by multiple factors.

As previously noted, 5mC marks do not show significant variance among vegetative cells, gametes, zygotes, or mt+/mt- cells, although their distribution is not random. They are concentrated (higher than 80%) at 23 loci with lower protein-coding gene density but enriched in repetitive sequences [24].

2.2 5mC writers

In photosynthetic organisms 5mC *de novo* mehtylation occurs via RNA-directed DNA methylation (RdDM) pathway, more extensively explained in Box 3. This pathway typically ends with DOMAINS REARANGED METHYLTRANSFERASE 2 recruitment. Any enzyme of this family has been found in *C. reinhardtii.* These "writers" enzymes can be classified as *de novo* or maintenance in four main families:

Dnmt1, Dnmt2, Dnmt3 and chromomethyltransferases (CMTs) [55]. The first three are common to plants and mammals, just in CpG methylation context, and CMTs are unique of photosynthetic organisms, responsible of CHG and CHH methylation.

As it was mentioned before, three different homologs of the Dnmt1/MET1 subfamily of DNA methyltransferases have been identified in *C. reinhardtii*: DMT1a (chloroplast located methyltransferase), DMT1b (mitochondrial located methyltransferase) and a DMT4 (Table I). While other microalgal species like *Chlorella sp*., *V. carteri* and flowering plant possess CMT3 homologue [56], *C. reinhardtii* does not appear to have this CMTs, these enzymes are less conserved in green algae [57]. Contrary to the expected, despite the absence of these CMTs, *C. reinhardtii* does exhibit this CHG modification in low amounts [28]; the enzyme responsible for this remains uncharacterized.

Recently it was demonstrated that *C. reinhardtii* contains a novel 5mC-modifying enzyme (CMD1) that is a TEN-ELEVEN TRANSLOCATION DIOXYGENASE (TET) homologue [58]. These enzymes promote DNA demethylation in mammals by a cascade of transformations of 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC5) in metazoans and fungi using 2-oxoglutarate (2-OG) as co-substrate [59]. Interestingly, this CMD1 requires L-ascorbic acid (vitamin C) as co-substrate transforming 5mC into 5-glyceryl-methylcytosines (5gmC) [60]. CMD1 deficient mutant cells showed hypermethylated and downregulated *LHCSR3* photoprotective gene, causing higher photo-oxidative damage under high light conditions, also proposing 5gmC as a new and independent potential epigenetic mark in eukaryotic DNA [58]. Further studies started to elucidate the molecular mechanism of this characteristic ten-eleven translocation enzymes (TET) homologue [61]. To our

196 knowledge, neither 5hmC, 5caC or 5fC modifying enzymes or presence of any of this
197 potential epigenetic marks have been characterized in this species.
198
199 ***2.3 N6-methyladenine***
200 In contrast, 6mA is found in *C. reinhardtii* DNA at an approximate ratio of ~0.4 mol%
201 6mA/A, present in nearly 84% of all genes (Figure 2) [47], which is comparable to the
202 ratio observed in its close relative, *Volvox carteri* [46]. This ratio is higher than that
203 observed in other microalgae species such as the protist *Nannochloropsis oceanica*
204 (~0.1% 6mA/A) [42] or in multicellular eukaryotes like mammals [62-64]. The
205 presence of this modification is substantially lower than in prokaryotic bacteria, where
206 6mA is the primary form of DNA methylation [48].
207 Recent evidence indicates that the quantity, function, and distribution of this epigenetic
208 mark are highly variable among species, with a lower presence in model plant genomes
209 such as *Arabidopsis* (~0.138-0.006% 6mA/A) [45], and different cultivars of *Oryza*
210 *sativa* (Japonica and Indica) [44]. In *C. reinhardtii*, 6mA exhibits a symmetrical
211 methylation pattern in ApT dinucleotides, suggesting the presence of methyltransferases
212 that maintain fully methylated DNA after replication. This pattern is only observed in
213 ciliated protozoa of the genera *Tetrahymena* and *Oxytricha*, and some fungal genera,
214 due to their sharing of the AMT1 clade of MT-A7 methyltransferases [65], [66].
215 Additionally, 6mA is enriched (~84%) around transcription start sites (TSS), showing a
216 positive correlation with active genes [47].
217 The addition of a methyl group at the N6 position of adenine slightly reduces base
218 pairing energy, potentially enhancing protein-DNA interactions [67]. Fu et al. (2015)
219 [47] proposed a regulatory model in *C. reinhardtii* in which 6mA marks the distribution
220 of nucleosomes by locating itself in the linker DNA between adjacent nucleosomes,

thereby determining the pattern of nucleosome positioning and potentially contributing to chromatin structures that enable the initiation of gene transcription (Figure 3A). This pattern was confirmed by restriction enzyme-assisted sequencing (DA-6mA-seq) [68] and single-molecule real-time sequencing (SMRT) [69].

Overall, the absence of evidence for a coordinated pattern between 5mC and 6mA in the *C. reinhardtii* genome suggests that there is no clear interplay between them, indicating that likely serve different functions and are regulated by distinct molecular pathways.

Box 2. Methyladenine mediates differential transcripts expression during *Chlamydomonas* life cycle.

m^6A plays an important role during *C. reinhardtii* sexual reproduction, being majority embedded in the 3´ UTRs regions (73%-76%) of its mRNAs and negatively correlated with the abundance of transcripts. Specifically, during mt+ gametogenesis, lower m^6A levels are observed at the *KIN9-3*, *DHC6*, and *KIN16-2* genes closely related to microtubule-based processes, changing dynamically during its different stages [70]. A few RNA m^6A methyltransferases candidates have been identified: CrMETTL3, CrMETTL14, CrMT1 and CrMT2; specifically, the first two show a significant decrease in their expression in gametes, and an increase doubling or even tripling their expression compared to vegetative cells during early zygote formation [70] (Table 1). As 6mA is the main DNA epigenetic mark it would be expected to show high variance through different stages of its life cycle. However, to our knowledge, no research has been carried out in this field and further investigation is required for assessing how is this mark involved in zygote formation and DNA inheritance.

2.4 N^4-methylcytosine

The study of N4-methylcytosine (4mC) has recently gained momentum in eukaryotic organisms. In bacterial systems, 4mC plays a pivotal role in restriction-modification systems, serving as a molecular tool to differentiate self-DNA from viral DNA, thereby safeguarding the former from degradation mediated by restriction enzymes [71]. In addition to 4mC, another modification, 6mA, was initially identified in prokaryotes as part of the same system [72], [73]. However, the presence of 4mC in the organellar and nuclear DNA of green algae remains largely unexplored across various species. To shed light on the existence of 6mA and 4mC in eukaryotes, a study conducted by O'Brow et al. (2019) [43] employed single molecule real-time sequencing (SMRTseq) and ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) to measure these epigenetic marks across 18 different genomes (comprising 2 prokaryotes and 16 eukaryotes, including *C. reinhardtii*). The results revealed that *C. reinhardtii*, along with the other 15 eukaryotic species, exhibited levels of 4mC that were below the limit of detection (LOD) <0.00005%. The apparent lack of this mark suggest that this specific mechanism has been lost in microalgae, however it may be still conserved at very specific loci. More efforts focused on this mark are needed for supporting previous hypotheses.

Enzyme	Family	Annotations	Domain(s)	Ref.	Accession number
DMT1a^{Cp}/DMT1 b^{Mt} (Near *MT* Locus)	Dnmt1	Coincidence with gamete and zygote cpDNA cytosine hypermethylation levels. Methylates any cytosines residues, no matter neighboring nucleotides. Lacking conserved motifs in the N-terminal region. DMT1a copy in mt- has a premature termination before domains	D-RFD and BAH	[24], [52]	Cre06.g249350_4532 / Cre06.g249500_4532
BDMT4^{Cp}	Dnmt1	Coincidence with gamete and zygote high cpDNA cytosine hypermethylation levels	D-RFD and BAH	[24]	Cre06.g286650_4532
METTL3	RNA-MTase	Lower expression in gametes (x0.5), higher expression for 1 day zygote (x3). Potentially methylates adenine	MT-A70	[70]	Cre06.g295600_4532
METTL14	RNA-MTase	Lower expression in gametes (x0.25), higher expression for 1 day zygote (x1.75). Potentially methylates adenine	MT-A70	[70]	Cre01.g050600_4532
CMD1	Ten-eleven DMT11 translocation (TET)	Modifies 5mC into 5gmC using vitamin C as co-substrate	2OGFeDO	[74], [75]	Cre12.g553400_4532
SET3	Histone-lysine N-methyltransferase	Methylation of the ninth lysin residue in histone H3. Related to tandemly repeated transgenes silencing. SU(VAR)3-9 homolog	SET	[76]	Cre02.g089200_4532
MUT9	Ser/Thr kinase	Phosphorylates histones H3 and H2A	Protein kinase	[77]	Cre16.g666200_4532
MUT11	WD40 repeat-like	Methylation at fourth lysin residue in histone H3	WD40	[78]	Cre08.g367650_4532
EZH	Enhancer of zeste Homolog	Methylates lysine 27 of histone H3. Induces transgene and transposon silencing. An ancestor element of multicellular eukaryotic (PRC2)	EZD2 and SET	[79]	Cre17.g746247_4532
Rubisco methyltransferase 2 (RMT2)	Chloroplast Methyltransferase Homolog	Potentially methylation, related to ameliorate damage by ROS, repair damage	Rubisco LSMT substrate-binding	[80]	Cre12.g524500_4532
Sir2-type histone	Sirtuins	Transgene-specific. Targets foreign DNA. Very different to SIRT6	Sir2	[81]	Cre10.g462200_

deacetylase (SRTA)		homolog in mammals. Mutated at UVM4 and UVM11 strains. NADH dependent			4532
DCL3	Dicer-like	miRNA cleavage	DEAD/DEAH box helicase, Dicer and Ribonuclease III	[82]	Cre16.g684715_4532
AGO3	Argonaute	Principal component of miRNA regulation of gene expression	Piwi, Argonaute linker 1 (PAZ)	[83]	Cre16.g689647_4532
DUS16	Double-stranded RNA binding protein	Interacts with CrDCL3 for miRNA cleavage	dsRNA-and ssRNA-binding motifs	[84]	Cre05.g232004_4532
MUT68	Terminal uridyltransferase	Uridylates at the 3' end of RNA inducing miRNA decay by exosomes	Nucleotidyltransferase	[85]	Cre06.g279474_4532

Table 1. Main *Chlamydomonas* characterized enzymes related to epigenetic mechanisms. BAH: bromo-adjacent homology; D-RFD: DNA methyltransferase replication foci domain; PAZ: Piwi/Argonaute/Zwille; EZD2: E(z) domain 2; SET: Su(var)3-9, Enhancer-of-zeste, Trithorax; Rubisco LSMT: Rubisco large subunit methyltransferase; Sir2: silent information regulator; Cp: in chloroplast; Mt: in mitochondria

3. *Histone Modifications and Chromatin remodeling*

Chromatin modifications are epigenetic regulatory features with major roles in various cellular events, yet they remain understudied in algae. In 1990, it was determined, using micrococcal nuclease digestion, that each *C. reinhardtii* nucleosome comprises a size of ~189 bp, more similar to mammalian cells than typical ~147 bp in plants. The nucleosome is wrapped around a histone octamer core, composed of two subunits of H2A-H2B dimer and two subunits of H3-H4 dimer, including the H1 linker histone [86]. Since then, significant discoveries have been made regarding this protein complex and its involvement in the regulation of gene expression through amino- and/or carboxy-terminal extensions or tails post-translational modifications (PTMs). During that time, the identification of the histones through their electrophoretic mobilities only allowed the differentiation of 1 band each histone and 2 bands for H2B. Nowadays, it is known there are more than 30 histone gene clusters through its genome containing between 32-34 copies of each gene. Those clusters are commonly flanked by bidirectional promoters, highly regulated at the epigenetic level, showing a very significant differential expression throughout most of the vegetative cycle (silenced) and a higher expression during the S/M phase [87]. Researchers Khan *et al*. (2018) [88] characterized with Top-Down Mass Spectrometry (TDMS) 13 proteoforms of the 4 core histones in this species, including 3 H2A variants, 2 H2B variants and an algae unique PTM: H4 Lys79 monomethylathion (H4K79me1). In recent research, Rommelfanger *et al*. (2021) [89], found 86 proteoforms by TDMS, and using a custom Python search script, of this 4 core histones and linker histone H1. When it comes to H2A, there are 5 canonical paralogs encoded by 26 genes with variations in their C-termini, along with the noncanonical H2A variant: H2A.Z. This latter variant is common in plants, such as H2A.X and H2A.W variants, neither of which are reported in *C. reinhardtii*. In

294 *Arabidopsis* and other plant species H2A.Z is usually found at the TSS, corelated with
295 high gene expression and H3K36me3 disposition; and with antagonistic H3K27me3 in
296 gene bodies of silenced genes [90]. In *C. reinhardtii* this variant undergoes multiple
297 acetylations at K6 and K19 residues and C-terminal truncation, while only about 20% of
298 canonical H2A proteoforms show PTMs [89]. Besides, there are more than 30 genes
299 across the *C. reinhardtii* genome encoding 16 potential H2B proteoforms. All of them
300 maintain an identical amino acid sequence up to residue 70, before their amino-terminal
301 tail, except for the noncanonical variant known as H2B.v1, which lacks the typical
302 amino-terminal extension found in H2Bs. Additionally, all *C. reinhardtii* H2B.v1 and
303 H2Bs exhibit trimethylation on the terminal alpha-amino group (Na*α*-me3) [89]. This
304 modification has been previously misidentified as Na*α*-ac in other studies due to their
305 similar mass and the absence of common mono- and dimethylation presence around it
306 [88]. Consistently, several studies have demonstrated the absence of acetylation or other
307 PTMs in H2B at any positions than the one previously mentioned [88], [89], [91]. The
308 histone H3 and its methylation marks are of particular interest as they are uniquely
309 distributed and dynamically changing during *C. reinhardtii* life cycle. Specifically, H3
310 trimethylation at fourth lysine residue (H3K4me3) is strongly associated (>80%) with
311 TSS and transcription termination sites (TTS) of actively gene expression, while TSS
312 genes deprived of H3K4me3 are significantly less expressed [92]. Around 120 loci,
313 including replication-dependent H2A/H2B and H3/H4 clusters, presents a strong
314 overexpression during S/M replication phase, coinciding with high peaks of H3K4me.
315 Out of TSS and TTS, most of H3K4 remain monomethylated (H3K4me1). Those
316 regions of *C. reinhardtii s* genome deprived of H3K4 methylation are enriched in 5mC
317 [92]. H3K9 monomethylation, H3K27 acetylation, mono- and dimethylation, and H3T3
318 phosphorylation are also histone modifications found at the promoter regions of silent

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319 genes in this species. Otherwise, 3 different H4 protein sequences have been detected.
320 The most abundant one (H4.1) is encoded by 30 genes, while the other 2 H4 variants are
321 each encoded by 2 different genes. This histone exhibits a distinctive multiple
322 acetylation pattern consisting of mono- to penta-acetylations at multiple lysine residue
323 positions, all of which share α-amino acetylation (Nα-ac). Similar to Arabidopsis and
324 other plants, *C. reinhardtii* does contain low levels of H4K20ac, which is an alternative
325 modification to strong H4K20 methylation observed in animals [89].
326 Posttranslational histone modifications may also be involved in the inheritance of
327 chromatin states. Histone phosphorylation, known for its role in long-term gene
328 silencing, was studied by Casas-Molado et al. (2008) [77] in a *C. reinhardtii* mutant
329 lacking a Ser/Thr protein kinase (MUT9p), which phosphorylates histones H3 and H2A.
330 This mutant displayed deficiencies in heritably repressing transgenes and transposons.
331 Chromatin immunoprecipitation analyses indicated an inverse correlation between
332 phosphorylated H3T3 and monomethylated H3K4 with di/trimethylated H3K4,
333 associating preferentially with silenced transcription units. Conversely, the absence of
334 these marks in mutants correlated with the reactivation of transgenes and transposons.
335

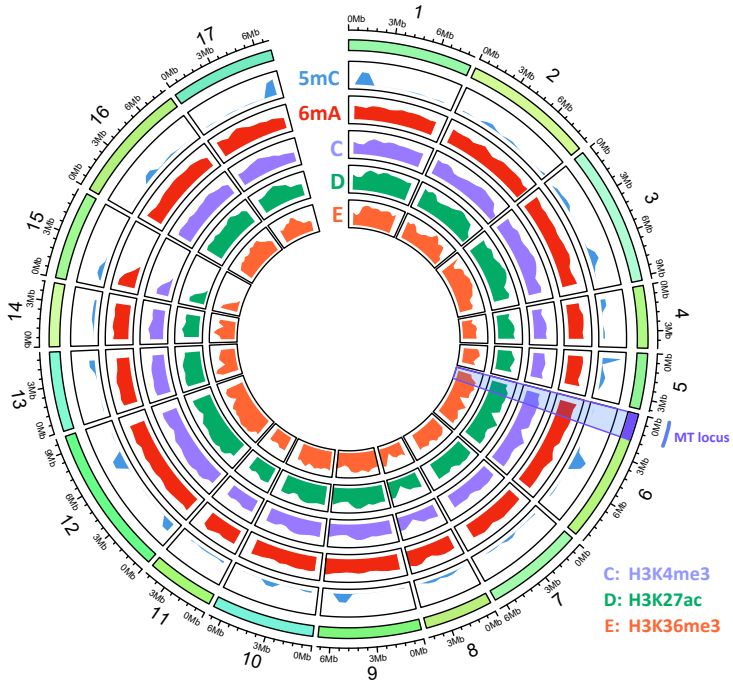


Figure 2. CIRCOS plot of *Chlamydomonas reinhardtii* epigenome elaborated employing Chlamytina beta version (https://github.com/RocesV/Chlamytina). Notice chromosomes representation corresponds to *C. reinhardtii* v6.1 genome while epigenetic marks represented were sequenced in older versions, some chromosomes as chr15 experimented a significant variance in their size.

4. small non-coding RNAs (ncRNAs)

Non-coding RNAs (ncRNAs) are RNA molecules that are not translated into proteins. Based on their size, ncRNAs are generally classified into long ncRNAs (lncRNAs, >200 nt) and small ncRNAs (sncRNAs, <200 nt). Major classes of sncRNAs include microRNAs. Recent advances have been made in understanding the complex roles of miRNAs in epigenetic regulation [93]. miRNAs, as epigenetic modulators, influence the

protein levels of target mRNAs without altering gene sequences. Furthermore, miRNAs can also be regulated by epigenetic modifications, including DNA methylation, RNA modification, and histone modifications. The reciprocal actions of miRNAs and epigenetic pathways seem to establish a miRNA-epigenetic feedback loop, exerting a significant influence on gene expression. The first unicellular organism in which miRNAs were described was *C. reinhardtii* in 2007, simultaneously by two different research groups [94], [95], when this intricate gene expression-regulation system had been studied exclusively from pluricellular complex organisms. *C. reinhardtii miRNAs* genes (*MIR*) are located at introns (~39%) and exons (~55%) of protein coding sequence genes [96]. This feature is more similar to animals than plants, in which *MIR* genes are embedded in the intergenic space.

Box 3. Summarizing miRNA-directed DNA methylation in *Chlamydomonas*

In plants, *de novo* and maintenance methylation are primary induced by RNA-directed DNA methylation pathway (RdDM) (Figure 3A). Generally, this highly conserved mechanism starts with the recruitment of RNA Pol IV by SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) and CLASSY (CLSY) into heterochromatin. RNA Pol IV is physically associated with RNA-DIRECTED RNA POLYMERASE 2 (RDR2) producing a double-stranded RNA (dsRNA) precursor chain (30nt to 45nt), which is subsequently recognized and processed by DICER-LIKE 3 (DCL3) endonucleases into short 20-25 nucleotide (nt) miRNAs [97]. In *C. reinhardtii*, the predominant fraction (28%) is 21nt miRNAs differing from typical 24nt miRNAs enriched portion related with higher plants RdDM pathway [96] (Figure 3B). Also, *C. reinhardtii* contains a unique RNA-binding protein DUS16 which plays a key role in miRNA production, located in the nucleus, and associated with unspliced mRNAs form and DCL3 [84]. Secondly, these 21nt dsRNAs are

methylated at their 3' ends by methyltransferase HUA ENHANCER 1 (HEN1), protecting them from degradation [98], and one strand binds with ARGONAUTE 4 (AGO4). This protein-miRNA duplex can identify and bind to complementary RNA "targets". These targets can be either mRNAs, resulting in post-translational silencing, or "scaffold" RNAs synthesized by RNA Pol V, resulting in DNA methylation. This binding leads to canonical RdDM machinery recruitment, including DNA methyltransferase enzyme DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), forming collectively what is called RNA-induced silencing complex (RISC) [99]. How this mechanism has evolved from unicellular eukaryotes is poorly understood and it entails multiple questions. In addition to the difference in the 21nt size predominance of *C. reinhardtii* miRNAs, this species presents 3 AGO and 3 DLC paralogs (being AGO3 and DCL3 the most relevant ones [82], [83]), different from the known homologs in plants. Multiple enzymes of the RdDM pathway such as HEN1, HASTY (responsible for transporting pre-miRNA out of the nucleus), DRM nor RDRs (key at dsRNAs generation from single stranded RNA) remain uncharacterized in this model microalgae.

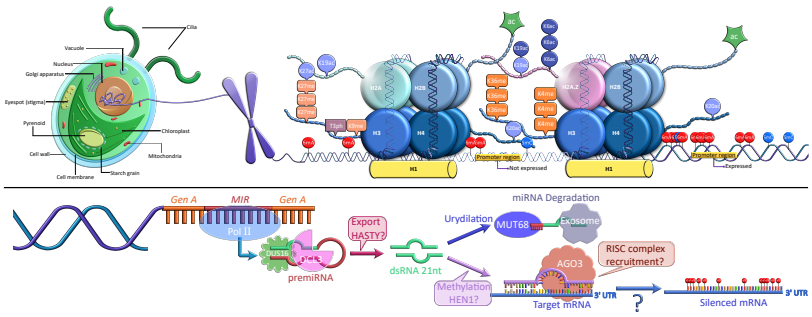


Figure 3. Diagram of how *Chlamydomonas* silencing machinery works. A. When promoter regions are deprived from 6mA, DNA would be compacted and not expressed. In contrast, a high presence of 6mA mark before this region and specific histone modifications and H2A.Z would induce gene expression. B. RdDM workflow as far we know.

There are multiple expression regulatory mechanisms mediated by miRNAs in higher plants, including miRNA-induced translation repression, blocking elongation or adenylation of target mRNA(s). In most plants, miRNAs need to be perfectly matched with their target. However, similarly to animals, *C. reinhardtii* small interfering RNAs (siRNAs) do not require perfect complementarity, thus, they may target multiple mRNAs. They bind to the 3' UTR, inducing inhibition once protein synthesis has started, thereby affecting the correct function or structural conformation of ribosomes [100]. A wide variety of miRNAs have been characterized in this species, demonstrating their involvement in biotechnological relevant processes such as lipid accumulation under nitrogen deprivation [101]. Recently, uncommon >26nt miRNAs associated with AGO1 were found to be upregulated also during nutrient deprivation [102]. While the presence of miRNAs in this species seems clear, there are two hypotheses regarding their origin: either there is, in fact, a non-canonical RDR yet to be characterized, or an alternative, unique miRNA biogenesis pathway exists in this species.

5. Crosstalk between epigenetic and epitranscriptomic mechanisms

In the last decade, epigenetics has started to turn its eyes towards RNA chemical modifications. These modifications have been known for long time, but recent development of methods allowing their study has led to the birth and establishment of

418 epitranscriptomics, studying RNA modifications, as a new discipline with its own
419 identity [103], [104]. Epitranscriptomics adds an extra regulatory layer to gene
420 expression regulation through epitranscriptomic plasticity.

421 Over 170 modifications have been described so far, involving all RNA types, including
422 mRNAs. RNA modifications are common throughout evolution [105] and the enzymatic
423 machinery responsible for at least the most studied modifications is evolutionary
424 conserved in eukaryotes [106], what suggests an also conserved and relevant role. In
425 general, these modifications determine mRNAs fate in eukaryotes, having been
426 described effects over all the processes encompassing their life from their synthesis to
427 their eventual translation or degradation, and even their transport inside and outside the
428 cell [107]. Furthermore, the interconnections between epigenetic and epitranscriptomic
429 mechanisms is starting to emerge [108]. Available studies have described
430 interconnections at different levels, like the effect of epitranscriptomic marks like m6A
431 over epigenetic machinery mRNAs fate or the role of the m6A reader YTHDC1 as a
432 scaffold for epigenetic machinery related to silencing as well as activation machinery
433 and involving histones as well lncRNAs [108].

434 Epitranscriptomic research in *C. reinhardtii* is lagged behind regarding that of higher
435 plants, especially crops and the model species *A. thaliana*, where the role of several
436 mRNA modifications in several developmental and stress response processes has been
437 described, including quantitative studies on the distribution and dynamism of mostly
438 m^6A, m^1A and 5mC and of the associated writer, eraser and reader proteins [109].

439 m^6A is the most prevalent and widely studied epitranscriptomic modification found in
440 eukaryotic mRNAs. It is found in transcript bodies and especially in 3'-UTRs around
441 TTSs, and it is involved in alternative splicing, nuclear export, stability, translation and

degradation. In *C. reinhardtii*, m6A is involved in sexual reproduction and life cycle [110]. Trancriptome-wide m6A methylomes, consistently with results obtained in other eukaryotes, identified enrichments of m6A in 3'-UTRs and of DRAC sequence motif. Moreover, m6A dynamism during sexual reproduction and life cycle seems to correlate negatively with gene expression and to be associated to transcripts related to energy metabolism and microtubule-based processes, critical for sexual reproduction.

Despite the initial steps of epitranscriptomic research in *C. reinhardtii* have been taken, outstanding research questions lay ahead. More modifications and under more situations must be studied. The identification of the enzymatic machinery responsible for them is another must and will represent the key to biotechnological applications. Furthermore, understanding the integration and interconnection of epitranscriptomic mechanism in the broader context of gene expression and chromatin biology, as well as cellular signaling networks, will allow designing more integral biotechnological applications.

6. Harnessing Epigenetic Insights for Innovative Productivity Strategies

Synthesizing and discussing the progress made in the comprehension of differential gene expression of this representative genus is crucial for understanding how the epigenetic mechanisms operate in eukaryotic organisms and developing new strategies to improve the manipulation of its adaptive capacity, leading to economically viable bioproducts and higher bioremediation rates in shorter times. Despite the development of a wide range of modern gene editing tools in model species such as *A. thaliana* over the last decades, these advancements have not led to equally controllable gene manipulation in *C. reinhardtii*, since the application of this knowledge in this species has historically encountered multiple issues.

Box 4. *Chlamydomonas* transgene silencing history

The lack of information about how transcriptional and post-translational highly efficient silencing machinery works in *C. reinhardtii* is closely related to the misunderstood failure of making new transformants of this species. Initially, transgene insertion and expression were more likely to be a random uncontrolled process. In 1997 researchers Cemtii *et al*. generated transformants with a copy of the eubacterial *aadA* gene conferring spectinomycin resistance inserted into the constitutive *RbcS2* gene [111], resulting in early repression and non-detectable *aadA* mRNA nor AADA protein. In 2000, a mutant (MUT-6) with a random disruptive insertion of *RbcS2:aadA:RbcS2* in *mut-6* gene (which encodes a DEAH-box RNA helicase), showed *aadA* expression reactivated, spectinomycin resistance and detectable transcript levels. But the more astonishing discover was Mut-6 had threefold higher transcription levels of the *transposon of Chlamydomonas 1* (*TOC1*) and increased transposition frequency of *TOC1* and *Gulliver* transposons [112]. Further research found two new *aadA* mutant strains deficient in epigenetic transgene silencing. The first one, Mut-9, was affected in a gene encoding a Ser/Thr kinase (MUT9) that phosphorylates histones H3 and H2A; and the other one, Mut-11, in a gene encoding a WD-repeat protein (MUT11), that form a complex with histone (H3, H2A, and H4) methyltransferase; they both were deficient in suppressing transposon activity and very sensitive to DNA-damaging agents [77], [78], [113]. Demonstrating histone marks and chromatin structure remodeling are the main factor inducing epigenetic silencing. H3K9me1 is particularly present in promoters of silenced transgenes [114], acting as a form of marking foreign DNA insertions. Nonetheless, disruption of *MET1* gene has also shown high levels of nuclear transgene expression [23]. This seems to be a complex mechanism mediated by the interaction of histone code and DNA methylation.

Nowadays, significant advancements and tools have emerged in this field, facilitating robust and efficient nuclear transgene expression. A promising strategy involves incorporating the *heat shock protein 70A* (*HSP70A*) promoter combined with *β2TUB*, *HSP70B* or *RBCS2* promoter, and optimal spaced at transgenic design. These fusion promoters induce a significant decrease of H3K9me1 levels, thereby counteracting epigenetic silencing mediated by heat shock factor (HSF1) [114]. Notably, Kaginkar et al. (2021) [115] developed a more effective artificial promoter composed of *PSAD* + *β2TUB2* promoters. For further information, a comprehensive compilation of *C. reinhardtii* transformation promoters and reporter genes is available [116].

Furthermore, two mutant strains, UVM4 and UVM11, exhibiting high efficiency in nuclear transgene expression, were generated in 2009 through UV mutagenesis [117]. Subsequently, these mutants have been extensively used in biotechnology research. It is noteworthy that both strains carry mutations in a highly conserved domain of a Sir2-type histone deacetylase (SRTA) transgene-specific, which is NADH-dependent. Dysfunction in this enzyme does not cause any affection at endogenous genes or TE behavior [81].

Commonly, various compounds such as 5-aza-2deoxycytidine, L-Ethionine, Zebularine, sodium butyrate and other histone deacetylases (HDAC) inhibitors are employed in several studies for investigating epigenetics. Very recently, Beauchemin et al. (2024) [118] achieved successful reversal of transgene silencing and higher transformant $mVenus^{+}$ in *C. reinhardtii* using HDAC inhibitors of the hydroxamate family, specifically suberoylanilide-hydroxamic-acid (SAHA). These results establish a new potential tool for recombinant production in this species and

516 microalgae in general. However, it is important to note that some of these drugs may
517 induce undesirable mutagenesis [16], [115].
518 In a different context, although artificial miRNA-induced gene silencing (MIGS)
519 has encountered several hindrances in its implementation in *C. reinhardtii* due to
520 our aforementioned limited knowledge of miRNAs biogenesis and how their
521 mechanisms of action work in *C. reinhardtii;* regulation of H_2, fatty acids, and
522 carotenoids has been successfully achieved [119], highlighting the utility of this
523 tool. Recently, the short tandem target mimic (STTM) RNA technique was
524 employed for targeted miRNAs suppression, marking *C. reinhardtii* as the first
525 unicellular organism undergo such testing. Despite its effectiveness, this technique
526 presents high nonspecifity, necessitating further elucidation of the optimal "spacer"
527 length for STTM compared to animals and multicellular plants [120].
528

529 **7. Epignetics is a new layer in microalgae stress biology**

530 Epigenetic modifications revealed as crucial in biological processes and stress
531 acclimation response all above the clades. Plants growth in dynamic environments, and
532 sessility make their development influenced by both biotic and abiotic factors [121]. In
533 addition to the various regulatory mechanisms to combat different stresses and to adapt
534 to the changing environmental conditions, plants also have the ability to memorize the
535 stress and even transmit it to the next generation. Despite the potential role of 6mA and
536 5mC in *C. reinhardtii* transcriptional regulation and therefore, in stress biology, only a
537 few studies about microalgae have been reported. Bacova *et al.* (2018) [27] described
538 that 40 µM of $CdCl_2 \cdot 5H_2O$ and 10 µM of 5-azacytidine (a DNA methyltransferase
539 inhibitor) and the combination of both modulate *C. reinhardtii* and *S. quadricauda*
540 biomass during 5 days of culture. Surprisingly, 5mC percentage remains constant under

all the treatments in *C. reinhardtii*, whereas is enhanced in *S. quadricauda*. These results suggest that the modulation of 5mC in *C. reinhardtii* through 5-azacytidine was not completely successful even though the modulation of the growth and some secondary metabolites as glutathione. In another study, Kronholm *et al.* (2017) [16] showed that reducing the 5mC and histone methylation variability with some epigenetic drugs (epi-drugs), reduce the adaption to salt, high CO_2 and phosphate starvation stresses in *C. reinhardtii*. Transgenerational epigenetic effects play a role in adaptive evolution and suggest that the relationship between changes in 5mC methylation patterns and differences in evolutionary outcomes, at least for quantitative traits such as cell division rates, is complex [16]. However, and even the 6mA are active marks, there is no study about how it contributes to stress response acclimation and finally adaption.

Only a few studies report changes in abundances of the histone writers (methyltransferases, acetyltransferases) and erasers (demethylases and deacetylases) in microalgae stress response. Colina *et al*. (2020) [122] depict the UV-C acclimation response, probably modulated by different epigenetic factors as HISTONE H3 lysine 27 (H3K27) demethylase and some JmjC proteins. In another study, Colina *et al.* (2020b) report that long-term acclimation is mediated by epigenetic mechanisms, being promising targets for further osmotic response characterization or exploitation towards engineering strains accumulating glycerol or other industry-demanding biomolecules. Ngan *et al.* (2018) [123] performed a genome wide histone modification and transcriptomic analysis to decipher the regulation of lipid biosynthesis in *C. reinhardtii* under N and S deprivation, respectively. They described 16 chromatin states, demonstrating an effective approach of using chromatin state changes to reveal potential regulatory components of lipid metabolism pathways in *C. reinhardtii* that can be applicable to other microalgae species. However, these studies only represent the top of

566 the iceberg on the knowledge about how histone PTMs regulate facultative
567 heterochromatin for stress acclimation in microalgae and further studies are required to
568 decipher microalgae biomolecule productivity.

569

570 **8. References**

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