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Loops, crosstalk, and compartmentalization: it takes many layers to regulate DNA methylation

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Abstract

DNA methylation is a conserved epigenetic modification associated with transposon silencing and gene regulation. The stability of this modification relies on intimate connections between DNA and histone modifications that generate self-reinforcing loops wherein the presence of one mark promotes the other. However, it is becoming increasingly clear that the efficiency of these loops are affected by crosstalk between pathways and by chromatin accessibility, which is heavily influenced by histone variants. Focusing primarily on plants, this review provides an update on the aforementioned self-reinforcing loops, highlights recent advances in understanding how DNA methylation pathways are restricted to prevent encroachment on genes, and discusses the roles of histone variants in compartmentalizing epigenetic pathways within the genome. This multilayered approach facilitates two essential, yet opposing functions, the ability to maintain heritable DNA methylation patterns while retaining the flexibility to modify these patterns during development.

Keywords

DNA methylation; histone modifications; histone variants; chromatin; self-reinforcing loops; RNA directed DNA methylation; chromatin readers; non-coding RNAs; heterochromatin

Introduction

In eukaryotic organisms, DNA is packaged within the nucleus via its associations with histone (H) proteins to form chromatin. The basic, repeating unit of chromatin is the nucleosome, a ball-like structure that contains two copies of H2A, H2B, H3, and H4, upon which approximately 150 bp of DNA is wrapped [1]. Despite this repeated nature, chromatin is not uniform. Its compaction status spans a continuum from the most accessible, euchromatic regions, which are enriched for expressed genes, to the least accessible,

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heterochromatic regions, which are enriched for silenced transposons and repeats. These different chromatin states are associated with specific histone variants [2] and with distinct modifications of DNA (*i.e.* cytosine methylation) [3] and/or the N-terminal tails of histone proteins (*e.g.* methylation, acetylation, ubiquitination, etc.) [4,5]—all of which are subject to variation depending on the cell-type or developmental stage [6,7]. These dynamics are facilitated by dedicated cellular machinery that modulate chromatin, which are broadly classified into four groups. “Writers” and “erasers” catalyze the addition and removal of specific DNA and histone modifications, respectively, “readers” bind these modifications, and “remodelers” alter the composition and/or position of nucleosomes within chromatin [8]. The resulting diversity in chromatin landscapes is critical for diverse biological processes as is evident by the numerous diseases and developmental defects associated with chromatin defects [6,9,10].

While some chromatin modifications are reset during DNA replication and cell division, others are maintained during mitosis, or even meiosis [11,12]. One such example is DNA methylation, where stable inheritance is central to its functions in regulating gene expression (to reinforce cellular identity) and silencing transposons (to prevent their mobilization within the genome) [3,13]. Like other chromatin modifications, DNA methylation patterns vary during development and in response to environmental cues [6,7]. This poses a conundrum of how stable inheritance is balanced with dynamic regulation to allow plasticity within DNA methylation pathways while avoiding potentially deleterious off-target methylation. Although DNA methylation is broadly conserved, the model plant *Arabidopsis thaliana* has proven an excellent system to gain mechanistic insight into its regulation. Unlike in mammals, most DNA methylation mutants are viable in plants. This properly, along with the stable inheritance of DNA methylation between generations, enabled the identification of several distinct pathways controlling DNA methylation in all three sequence contexts, CG, CHG and CHH (H=A, T, or C) via forward and reverse genetic approaches [3,13]. In this review, we highlight recent work, mainly from Arabidopsis, that has broadened our view on how DNA methylation patterns are maintained by self-reinforcing loops, how the boundaries of DNA methylation are reinforced to prevent aberrant methylation patterns near genes, and how histone variants gate access to heterochromatin to regulate global DNA methylation patterns.

DNA methylation patterns are maintained via self-reinforcing loops

Characterization of the mechanisms controlling the establishment and maintenance of DNA methylation in plants and animals have revealed a common theme—while the proteins and pathways are not always conserved, they all rely heavily on self-reinforcing loops, herein defined as regulatory circuits that connect and promote the maintenance of specific chromatin modifications. In this section we provide an overview of these loops, separated by sequence context, and highlight recent findings, focusing mainly on plants (Fig. 1).

CG methylation is faithfully inherited during DNA replication by the coordinated activities of DNA methyltransferases [METHYLTRANSFERASE 1 (MET1) in plants and DNA methyltransferase 1 (DNMT1) in mammals] and methyl-DNA binding proteins [VARIANT IN METHYLATION 1–3 (VIM1/VIM2/VIM3) in plants and Ubiquitin-like with PHD and

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RING Finger domains 1 (UHRF1) in mammals] [14] (Fig. 1a). Briefly, this self-reinforcing loop was initially proposed based on the specificity of the VIMs and UHRF1 for hemi-methylated DNA, which recruits MET1 or DNMT1, respectively, to newly replicated DNA to restore CG sites to a fully methylated state [14]. However, the aforementioned proteins contain additional chromatin binding domains (Fig. 1a), which remain poorly understood in plants. In mammals, the binding preferences of additional reader domains in UHRF1, as well as several genes targeted for ubiquitylation via its RING domain, have been identified. These components together reinforce the targeting of DNMT1 to replicating DNA (Fig. 1a) [14]. In addition, more recent work uncovered a UHRF1-independent mechanism of DNMT1 targeting. This mechanism is critical for maintaining CG methylation in heterochromatin via the H4K20me3 and H3K9me3 binding activities of its bromo-adjacent homology (BAH) and the replication foci targeting sequence (RFTS), respectively [15,16] (Fig. 1a). As the VIMs and MET1 possess similar domains as UHRF1 and DNMT1 (Fig. 1a), it will be interesting to learn if these domains fulfill a similar or divergent function in plants.

In plants, non-CG methylation is maintained in pericentromeric heterochromatin by three related H3K9 methyltransferases, SU(VAR)3–9 HOMOLOG 4–6 (SUVH4/SUVH5/SUVH6), and one of two CHROMOMETHYLASES (CMT), CMT2 for CHH or CMT3 for CHG methylation [17]. These factors generate self-reinforcing loops that rely on the reciprocal specificities of their “reader” and “writer” domains (Fig. 1b). Like their counterparts in animals, yeast, and drosophila, all three SUVHs contain histone methyltransferase domains that catalyze H3K9 methylation via their SET domains [17,18]. However, these plant SUVHs also contain SET and RING-ASSOCIATED (SRA) domains that bind methylated DNA allowing them to link histone and DNA methylation [17]. Conversely, CMT2 and CMT3 possess BAH and Chromo domains that bind H3K9 methylation and DNA methyltransferase domains largely specific for CHH and CHG methylation, respectively [17]. Before the characterization of AGENET DOMAIN (AGD)-CONTAINING P1 (ADCP1), which revealed similarities to Heterochromatin Protein 1 (HP1) in animals [19] based on its roles in binding H3K9 methylation, condensing chromatin, and facilitating silencing [20–22], the aforementioned SUVH and CMT factors were thought to be sufficient to maintain non-CG methylation in heterochromatin (Fig. 1b). However, *adcp1* mutants have reduced H3K9me2 and non-CG methylation at heterochromatic loci normally targeted by these SUVHs and CMTs, revealing another layer of epigenetic regulation [21,22]. Notably, the impact on DNA methylation is weaker than on H3K9me2 [21,22], possibly due to the persistence of H3K9me1 in *adcp1* mutants [21]. In moving forward, it will be important to determine the mechanism(s) employed by ADCP1 to reinforce the connections between DNA and histone methylation, chromatin compaction, and transcriptional silencing.

As observed in various animals and fungi, plants also utilize non-coding RNAs to target DNA methylation to transposons [23]. In plants, this pathway, termed RNA directed DNA Methylation (RdDM), establishes DNA methylation in all sequence contexts and maintains non-CG methylation in euchromatin (Fig. 1c) [24–26]. RdDM employs two plant-specific RNA polymerases, Pol-IV and Pol-V, that are recruited to chromatin via self-reinforcing loops to generate small interfering RNAs (siRNAs) and long noncoding

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RNAs, respectively. Together, these non-coding RNAs guide the de novo methyltransferase, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), to transposons and repeats (Fig. 1c). Briefly, Pol-IV targeting is linked to H3K9 methylation through its interaction with SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1), a methyl-H3K9 reader required for siRNA production and methylation at many RdDM targets [24–26] (Fig. 1c). Pol-V targeting is dependent on two DNA methylation readers (SUVH2 and SUVH9) as well as the end-product of the RdDM pathway: DNA methylation [25,26] (Fig. 1c). Recent work has added to our understanding of both these self-reinforcing loops. For Pol-IV, Parent et al. [27] provide genomic and cytogenetic evidence that in addition to SUVH4, 5 and 6, SUVH9, and likely also SUVH2, are required for H3K9 methylation and efficient RdDM specifically in early embryo development. Although previously thought to be inactive [28], this function requires a key catalytic residue in the SUVH9 histone methyltransferase domain [27]. Thus, depending on the tissue or developmental stage, either SUVH9 and 2 and/or SUVH4, 5 and 6 are required to deposit H3K9 methylation and facilitate Pol-IV recruitment by SHH1 (Fig. 1c). For the Pol-V loop, recent work confirmed that the MET1 and CMT3 pathways back-up methylation at many RdDM locations to ensure efficient Pol-V targeting [29] (Fig. 1c). Taken together, these findings reveal how tissue-specific redundancies within family members and cross-talk between DNA methylation pathways work together to ensure efficient RdDM.

Local and global mechanism to maintain DNA methylation homeostasis at TEs within euchromatin

Despite their interconnectedness, the pathways for maintaining DNA methylation in plants primarily regulate different parts of the genome: MET1 maintains CG methylation throughout the genome [3,14], CMT2 and CMT3 maintain non-CG methylation at long TEs enriched in pericentromeric heterochromatin, and DRM2 maintains CHH and CHG methylation at the edges of long TEs and at shorter TE fragments near genes in the chromosome arms with some aid by CMT3 [30,31] (Fig. 2a). Below we highlight recent insights into how these patterns of non-CG methylation are reinforced on local (*i.e.* locus by locus) and/or global scales (*i.e.* affecting many loci) to ensure proper DNA methylation homeostasis.

As the RdDM pathway acts primarily at the boundaries of heterochromatin and euchromatin, there are several mechanisms to keep this pathway in check and prevent aberrant silencing of genes that neighbor TEs. One such mechanism involves a family of methyl-cytosine specific DNA glycosylates, including REPRESSOR OF SILENCING 1 (ROS1), DEMETER-LIKE 2 (DML2), and DML3, herein abbreviated as RDD, that antagonize the RdDM pathway in somatic tissues by removing methylation at thousands of loci, most of which are near genes [32] (Fig. 2a). Recently, roles for a fourth family member, DEMETER (DME), in this process were revealed by using genetic tricks to bypass its essential role during female gametogenesis [33]. While this *dme* mutant only has minor effects on its own, it strongly enhances the *rdd* phenotype, with *drdd* mutants affecting more loci and causing additional hypermethylation, including at the *FLOWERING LOCUS C (FLC)* gene, which regulates flowering time (Fig. 2b) [33]. Beyond antagonizing RdDM in promoter regions, these

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demethylases were found to play new roles in removing CG methylation from the coding regions of ~1000 genes, of which some showed altered expression [33]. Notably these genes are largely distinct from the previously identified gene body methylated (gbM) loci [34], and their methylation appears to be more dynamic during plant development [33]. Thus, in addition to its roles in reproduction, DME also plays an important role in somatic tissues by pruning DNA methylation in gene bodies and at RdDM targets. Another mechanism that keeps methylation near genes in check functions on a global scale by dampening the demethylation pathway when RdDM is defective. This mechanism, dubbed a methylation rheostat, controls the expression of *ROS1*, the demethylase that affects the most loci when mutated alone [32,35,36]. Surprisingly, RdDM-mediated methylation of the *ROS1* promoter is required to promote, rather than repress *ROS1* expression, preventing further decreases in methylation when RdDM is defective (Fig. 2c). As *ROS1* is also downregulated in maize RdDM mutants [37,38], the interactions between these pathways appear to be a conserved mechanism to ensure that the methylation and demethylation pathways remain in balance.

As recently reviewed in [24], yet another mechanism to control where the RdDM pathway is active is through regulating the targeting of Pol-IV (Fig. 1c). This regulation also occurs on both local and global levels and is mediated by Pol-IV associated proteins, including four putative chromatin remodeling factors, CLASSY (CLSY) 1–4 [39], and two chromatin readers, SHH1 [40,41] and ZINC FINGER, MOUSE DOUBLE-MINUTE/SWITCHING COMPLEX B, PLUS-3 Protein (ZMP) [42] (Fig. 1c). Briefly, the four CLSYs control the locus- and tissue-specific targeting of Pol-IV to generate distinct DNA methylation patterns via a combination of their tissue-specific expression patterns and their reliance on distinct chromatin features [39,43,44]. In all tissues tested, CLSY1 and CLSY2 target Pol-IV to short euchromatic TEs by bridging the interaction between the H3K9-methylation reader SHH1 and the Pol-IV complex as part of a self-reinforcing loop [39,43] (Fig. 1c). CLSY3 and CLSY4 target Pol-IV to pericentromeric heterochromatin by poorly understood mechanisms that differ between tissues, with, for example, a partial reliance on CG methylation in flowers [39] and connections to a conserved DNA motif in ovules [43] (Fig. 1c). In addition, ZMP may also act in connection with CLSY3 and CLSY4 as it also promotes Pol-IV activity in pericentromeric heterochromatin [42]. Notably, the binding preferences of SHH1 and ZMP, which are both selective against H3K4 methylation marks associated with expressed genes [41,42,45] (Fig. 1c), may serve the dual role of recruiting Pol-IV to transposons while also helping to prevent RdDM activity near genes. Indeed, several H3K4 histone demethylase mutants show increased H3K4me2/3 levels and reduced siRNAs and DNA methylation levels at gene-proximal loci regulated by SHH1 [46]. These findings suggest that deposition of H3K4me during transcription protects promoters from Pol-IV transcription by blocking SHH1 binding [46] (Fig 1c). Highlighting the significance of precise Pol-IV targeting, disrupting the balance between targeting factors, such as in the *zmp* mutant or specific *clsy* mutants, results in both losses and gains in methylation [42,44]. This observation is consistent with a global redistribution of Pol-IV and/or altered interactions between the RdDM and demethylation pathways [24].

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Roles for histone variants in regulating access to heterochromatin and compartmentalizing the RdDM and CMT pathways.

The conserved H1 linker histone and a plant specific H2A variant, H2A.W, have emerged as gatekeepers of heterochromatin. They generate just the right level of compaction to restrict access to epigenetic regulators, including the DNA methylation machinery, but still allow access via the activities of chromatin remodeling factors and chaperones (Fig. 3). In this section we highlight the following aspects: the distinct effects of H1 and H2A.W on chromatin compaction [47–51], the role of H1 in compartmentalizing the genome into regions where non-CG methylation is controlled by different methyltransferases [31,51–53], and the mechanisms facilitating access to regions marked by H1 and H2A.W [31,48,52,54,55].

Consistent with their roles in promoting chromatin compaction, both H1 and H2A.W are enriched in heterochromatin, including at cytologically visible foci named chromocenters, that are marked with high levels of DNA methylation and H3K9 methylation [47,49–51,56,57]. Furthermore, ectopic expression of H1 or H2A.W in cells with de-compacted chromocenters is sufficient to drive chromocenter formation [47,48]. For H2A.W, this function depends on its conserved C-terminal tail [47]. However, these histone variants play distinct roles in shaping heterochromatin. In *h1* mutants, chromocenters are largely dispersed [48–51], with only small nanodomains remaining [49] (Fig. 3a). Yet they retain their heterochromatic properties as they are still marked with H3K9 methylation [48,49] and DNA accessibility assays show relatively minor increases [49–51,53] (Fig. 3a). Meanwhile, in *h2a.w* mutants, chromocenters are larger and, unexpectedly, show decreased DNA accessibility [47,51] (Fig. 3a). To understand why chromatin is less accessible when a protein that promotes chromatin compaction is mutated, the profiles of various epigenetic features were assessed in *h2a.w* mutants, revealing increased levels of H1 at heterochromatin [51]. This finding suggests that H2A.W antagonizes H1 deposition, possibly via competition for binding to linker DNA via its C-terminal domain, to prevent excessive levels of H1 at heterochromatin [51]. Together these findings support a model where altering the ratios of these histone variants tunes the accessibility of heterochromatin, with H1 decreasing accessibly and H2A.W increasing accessibility, which, as discussed below, has a profound effect on DNA methylation patterns.

Ever since the observation that DRM2 and the RdDM pathway act primarily at short TEs in the chromosome arms and at the edges of long TEs in pericentromeric heterochromatin, while CMT2 and CMT3 act primarily in the bodies of long TEs, the question of how these pathways, that both rely on H3K9 methylation, are compartmentalized within the genome has loomed large [30,31]. However, recent studies have shed light on this question by demonstrating that H1 is a barrier to RdDM, effectively restricting RdDM activity to short TEs (Fig. 3a, b). First, global profiling revealed a positive correlation between H1 enrichment and TE length, establishing a gradient with lower levels at shorter, more euchromatic TEs regulated by RdDM and higher levels at longer, more heterochromatic TEs regulated by the CMT pathways [50,51]. Second, modeling experiments revealed H1 as the best predictor (~12% error rate) of regulation by the CMT over the RdDM pathways [53].

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Finally, in *h1* mutants, long TEs have increased siRNAs and DNA methylation [31,52,53]. These increases are positively correlated with wild-type H1 levels [52,53] and are biased towards linker DNA [53]. Furthermore, the hypermethylation remained in *h1cmt2* mutants, confirming its dependence on the RdDM pathway [53]. Thus, in the absence of H1, RdDM can invade the territory normally controlled by the CMTs.

Interestingly, genetic approaches to determine what features are required for the redistribution of RdDM in *h1* mutants revealed a reliance on non-CG methylation (mediated redundantly by CMT2 and CMT3) but not on H3K9 methylation [53]. Further supporting this conclusion, redistribution also does not require CLSY1, CLSY2, or the H3K9 methylation reader SHH1, and thus likely relies on targeting by CLSY3 and CLSY4 or employs a novel targeting mechanism [53] (Fig. 3b). Regardless, the redistribution of RdDM throughout the TE-dense pericentromeric heterochromatin in *h1* mutants results in concomitant reductions in siRNAs and DNA methylation at shorter TEs normally regulated by RdDM [31,52,53] (Fig. 3b). Notably, the opposite redistribution is observed in *h2a.w* mutants, as DNA methylation levels are increased at RdDM targets and decreased in pericentromeric heterochromatin [51] (Fig. 3b). The former demonstrates that H2A.W limits RdDM at short TEs and the latter suggests that the increased H1 levels, and decreased accessibility, observed at heterochromatin in *h2a.w* mutants further restricts access to the DNA methylation machinery [51]. Taken together, these findings demonstrate the importance of establishing the proper compositions of heterochromatin to maintain DNA methylation homeostasis.

Although RdDM is efficiently excluded from H1-enriched heterochromatin, these regions are normally modified by other methylation pathways. Here we highlight several mechanisms, involving chromatin remodeling factors or developmentally programmed depletion of H1, that allow selective access to heterochromatin. The DECREASED DNA METHYLATION 1 (DDM1) remodeler was the first factor linked to overcoming the H1 barrier [31] and both DDM1 and its mammalian ortholog, Lsh1, were subsequently shown to provide DNA methyltransferases access to nucleosomal DNA in addition to linker DNA [58] (Fig. 3b). These discoveries stemmed from observations that combining *ddm1* with RdDM mutants (e.g. *drd1*) resulted in a nearly complete loss of methylation in all sequence contexts, demonstrating that MET1, the CMTs, and, to a much lesser extent, DRM2, depend on DDM1 [31]. In searching for the heterochromatin feature blocking access to these DNA methyltransferases, H1 was identified as the culprit since DNA methylation levels are largely restored in *ddm1,h1* double mutants and this restoration followed the distribution of H1 (*i.e.* restoration was stronger in heterochromatin and weaker in euchromatin) [31]. Together, these findings demonstrate DDM1 plays a critical role in providing DNA methyltransferases access to H1-marked heterochromatin in vegetative tissue (*i.e.* leaves).

During other stages of development, additional mechanisms are employed to allow the methylation machinery access heterochromatin. For example, different strategies are employed on the male and female sides to grant the DME demethylase access to heterochromatic target loci and facilitate epigenetic reprogramming during reproductive development. On the female side, the FACT (facilitates chromatin transactions) remodeling

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complex is required for demethylation at ~1/2 of DME target loci [54], including at several maternally imprinted genes [54]. These FACT-dependent loci are enriched at long TEs in pericentromeric heterochromatin [54] (Fig. 3c) and demethylation is restored in *h1* mutants at ~10% of these targets [54]. Thus, while the full scope of FACT-dependent activities promoting demethylation remain to be determined, one key function is to facilitate DME access to H1-enriched heterochromatin [54]. On the male side, FACT is not required [54] and instead naturally low expression levels of H1 in the vegetative cell of the pollen grain grant DME access to the most heterochromatic regions of the genome [48] (Fig. 3d). Indeed, when H1 is ectopically expressed in the vegetative cell, DME targets gain methylation and the plants are less fertile, attesting to the importance of properly regulating DNA methylation patterns in this cell type [48]. Finally, embryogenesis was recently discovered as another developmental window where the methylation machinery, in this case RdDM, has more access to long-TEs in pericentromeric heterochromatin [52]. While it remains unclear if this access is mediated by alteration in H1 levels, remodeling activity, or another mechanism, siRNA profiles during embryogenesis resemble *h1* leaves, demonstrating that regions of the genome enriched in H1 in post-embryonic tissues are more accessible to RdDM during embryogenesis [52].

Conclusions

Building upon the localized connections between chromatin readers and writers that were initially discovered to reinforce the maintenance of DNA methylation, this review highlights how our view of the mechanisms regulating DNA methylation have expanded to include additional layers. Extensive cross-talk between pathways has emerged as a mechanism to provide critical checks and balances between DNA methylation pathways to maintain DNA methylation homeostasis and protect genes from aberrant methylation and silencing. In addition, histone variants and heterochromatin compaction states have arisen as key factors that compartmentalize the genome and allow largely independent regulation of euchromatic versus heterochromatic transposons and repeats. Nonetheless, important questions remain across all these scales of regulation. For the self-reinforcing loops, several targeting questions remain including whether MET1, like DNMT1, can be targeted to heterochromatin outside of replication, how ADCP1 reinforces methylation by the CMTs, and if CLSY3 and CLSY4 also participate in self-reinforcing loops. Regarding the balance between methylation and demethylation, it remains unclear how disrupting Pol-IV targeting short circuits this system to cause both gains and losses of methylation. In addition, many questions related to the tissue specific regulation remain. For example, if and how the MET1 and CMT pathways, like the RdDM pathway, might be modulated during somatic development and what other developmental stages will be added to the growing list of cases in which modulation of histone variants impacts global methylation patterns. Finally, it remains unclear why DDM1 is able to provide the MET1 and CMT pathways, but not the RdDM pathway, access to H1-enriched heterochromatin. Perhaps this is due to the requirement of transcription by Pol-IV and Pol-V? With increasing access to tools and techniques to simultaneously profile multiple chromatin features on the level of specific tissues, organs, and even single cells, the field is now well poised to address this next wave of questions. Given the conservation of DNA methylation pathways, understanding

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the mechanisms shaping DNA methylation patterns in a model system like Arabidopsis will not only advance approaches for epigenetic engineering in plants, but may also provide key insights of relevance in other species where precise epigenetic changes in methylation can be leveraged to control important biological traits.

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•• of outstanding interest

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Highlights

- Chromatin readers and writers shape methylation patterns via self-reinforcing loops
- Local and global mechanisms balance DNA methylation pathways to maintain homeostasis
- Histone variants affect chromatin access and compartmentalize methylation pathways
- Chromatin remodelers promote DNA methylation and demethylation in heterochromatin.

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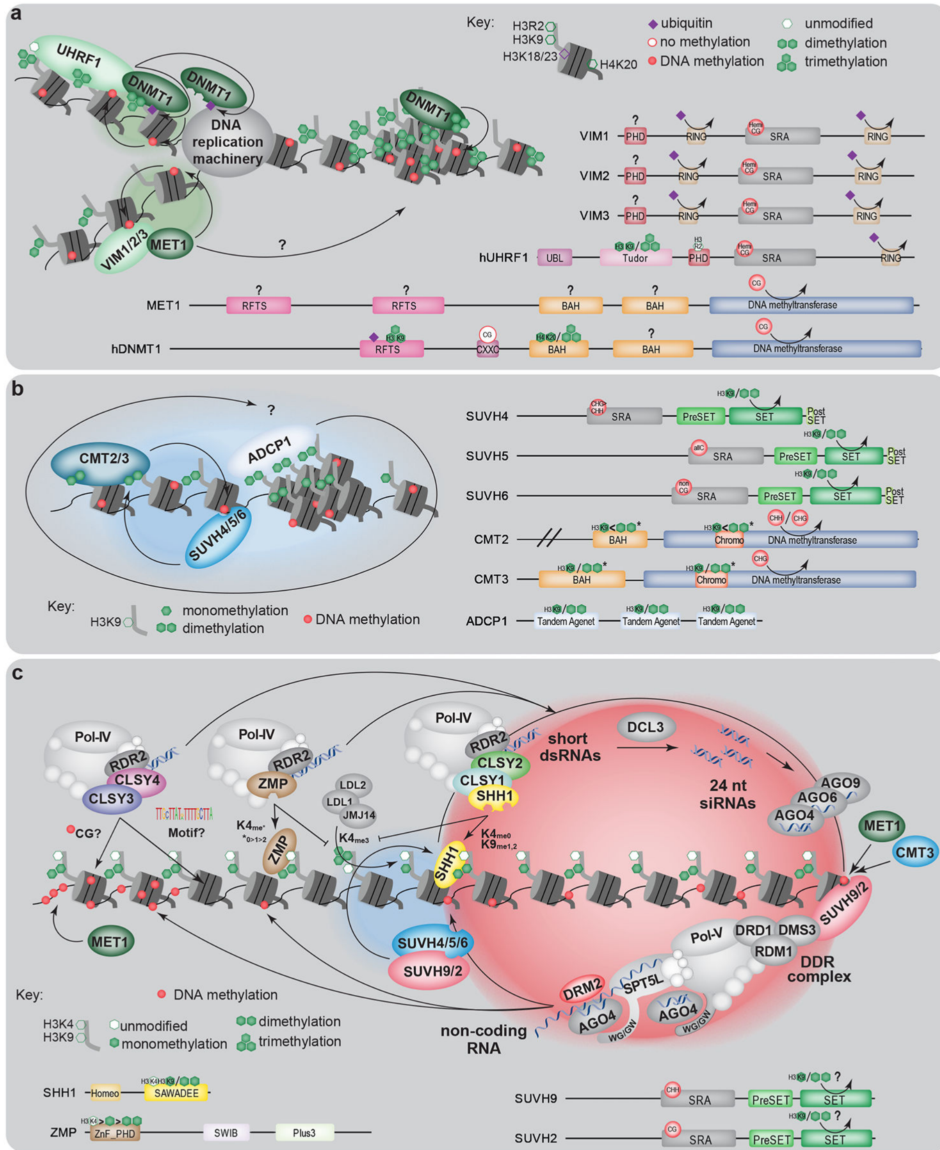

Figure 1. Self-reinforcing loops involving different combinations of reader and writer proteins maintain DNA methylation in the CG, CHG, and CHH contexts.

For all panels, the self-reinforcing loops are highlighted using different colored circles, the main histone and DNA modifications are as indicated in the key, and the gene models for the readers and writers are shown to scale, with the exception of CMT2, where the missing portion is marked by the double forward slash marks. All models are from Arabidopsis except in **a**, where human proteins are prefixed with an “h”. The specificities of each reader and writer are shown above the domain and the residue or methylation context are included. Unknown specificities are marked with question marks, dual specificities are separated by a slash, and preferences are shown with greater or less than symbols. For the CMTs, the asterisk indicates preferences determined for the combined rather than individual reader domains. **a.** Maintenance of CG methylation during replication in plants (lower fork) and mammals (upper fork). The conserved reader proteins, the VIMs and UHRF1, respectively, bind hemi-methylated CG methylation to recruit the MET1 and DNMT1 methyltransferases,

respectively, to newly replicated DNA to generate fully-methylated DNA (green shaded circle). In mammals, these associations are reinforced by the H3R2 reader and ubiquitination writer activities of UHRF1 as well as the reader activities of the DNMT1 RFTD domain, all of which remain poorly understood in plants. DNMT1 is also recruited to heterochromatin in a UHRF1-independent manner via its BAH domain's interaction with H4K20 methylation. **b.** Maintenance of CHH and CHG methylation in connection with H3K9 methylation by the reciprocal reader and writer specificities of the depicted CMT and SUVH proteins: The CMTs read H3K9 methylation and write DNA methylation while the SUVH proteins do the opposite (darker blue shaded circle). These modifications are reinforced by the multivalent H3K9 methyl-binding activity of ADCP1 via a mechanism that remains unclear (lighter blue shaded circle). **c.** Establishment of DNA methylation in all sequence context and maintenance of non-CG methylation by the RdDM pathway, involving two self-reinforcing loops (blue and red shaded circles). Pol-IV is recruited to chromatin by the CLSY proteins, which differ in their targeting mechanisms. CLSY1 and CLSY2 target Pol-IV via SHH1, which reads the H3K9 methylation deposited by two sets of SUVH proteins that are themselves recruited to chromatin by DNA methylation (blue circle). Once targeted, Pol-IV and RDR2 generate short double stranded RNAs (dsRNAs) that are processed into 24 nucleotide (nt) siRNAs by DCL3 and loaded into AGO proteins. These AGO complexes bind the WG/GW repeats in Pol-V and to its nascent non-coding RNA transcripts resulting in the recruitment of DRM2 and the deposition of methylation in all sequence contexts. Pol-V associates with the DDR complex and is recruited to chromatin by SUVH9 and SUVH2 via their methyl-DNA binding activities forming a positive feedback loop wherein DNA methylation by RdDM, or by CMT3 and MET1 at some loci, promotes more methylation. CLSY3 and CLSY4 target Pol-IV to another set of loci that are not subject to the same self-reinforcing loops and rely on different chromatin features (*i.e*. CG methylation by MET1 or a DNA motif) depending on the tissue. Finally, the reader activities of both ZMP and SHH1 are selective against H3K4 methylation which, along with the activities of the JMJ14, LDL1, and LDL2 H3K4 demethylases, may help balance the activity of RdDM near genes.

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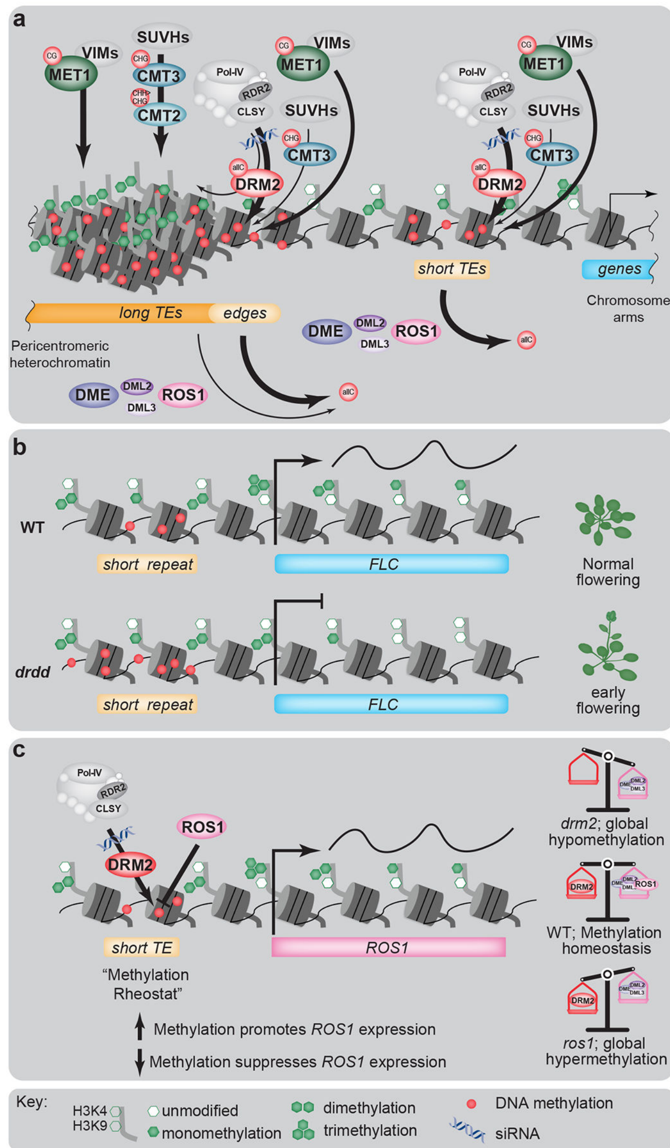

Figure 2. Compartmentalization of the pathways controlling DNA methylation within the genome.

a. The relative activities of DNA methylation (upper) and DNA demethylation (lower) pathways at long TEs in pericentromeric heterochromatin versus short TEs near genes in the chromosome arms. The thickness of the arrows scale with the contributions of the pathways and for the demethylases so does the size of the proteins. MET1 plays a major role in maintaining CG methylation in all regions. CMT2 and CMT3 maintain most of the non-CG methylation at long TEs while RdDM plays the major role at the edges of long TEs and at short TEs near genes, with some back up by CMT3. The demethylases antagonize the activities of RdDM at long TE edges and short TEs, with ROS1 and DME playing the major roles in vegetative and reproductive tissues, respectively. **b.** Example of a locus, *FLC*, that is hypermethylated and silenced in the demethylase quadruple mutant, *drdd*, resulting in early flowering. **c.** Depiction of a methylation rheostat that locally controls *ROS1* expression (left) and globally controls the balance between methylation and demethylation pathways (right).

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Normally, DNA methylation by RdDM, which paradoxically promotes *ROS1* expression, is balanced with demethylation by ROS1, which suppresses its own expression resulting in methylation homeostasis. In RdDM mutants (*e.g. drm2*) non-CG methylation is globally reduced, including at the *ROS1* locus, which reduces *ROS1* expression and limits the amount of demethylation to preserve what methylation remains. Alternatively, in *ros1* mutants, or in the *rdd* triple or *drdd* quadruple mutants, the genome becomes increasingly hypermethylated due to the accumulation of methylation at RdDM targets. The main histone and DNA modifications for all panels are as indicated in the key at the bottom.

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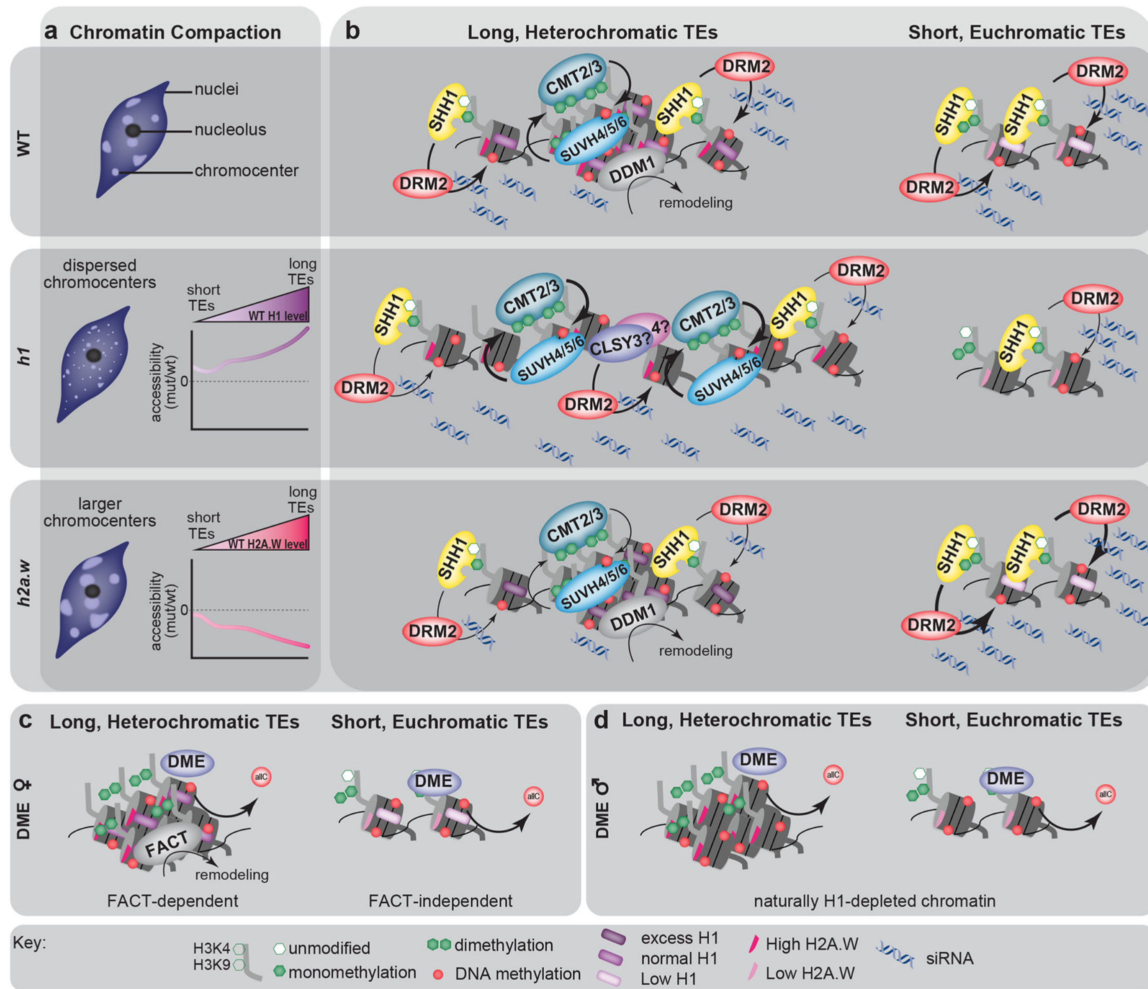

Figure 3. The roles of histone variants in regulating chromatin compaction and accessibility to different methylation pathways.

a. Chromatin compaction and accessibility in wild-type (WT), *h1*, and *h2a.w* plants. The main features of a WT nuclei are labeled and alterations are shown for *h1* and *h2a.w*. On the right, changes in chromatin accessibility compared to WT controls are graphed relative to TE size, which is positively correlated with H1 and H2A.W levels in WT plants. **b.** Cartoons depicting the effects of H1, H2A.W and DDM1 on activities of the RdDM and CMT pathways at long versus short TEs. Compared to WT, *h1* mutants have more open chromatin at long TEs, eliminating the need for DDM1 and simultaneously increasing the activities of the CMT and RdDM pathways at long TEs while sequestering RdDM away from short TEs. In *h2a.w*, H1 levels are higher at long TEs and access to both the CMT and RdDM pathway are reduced. However, the opposite is observed at short TEs where RdDM has more access in the absence of H2A.W. **c** and **d.** Mechanisms employed during reproductive development to allow DME access to long TEs involving either the remodeling activity of the FACT complex on the female side in **c**, or reduced *H1* expression resulting in H1-depleted heterochromatin on the male side in **d**. The main histone and DNA modifications for all panels are as indicated in the key at the bottom.