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Current Opinion in Plant Biology

Crosstalk among pathways to generate DNA methylome

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Abstract

Cytosine is methylated in both CpG and non-CpG contexts (mCG and mCH, respectively) in plant genomes. Although mCG and mCH are almost independent in regard to their "maintenance," recent studies uncovered crosstalk between them during their "establishment," which unexpectedly functions in both RNAi-dependent and -independent pathways. In addition, the importance of linker histone H1 and variants of histone H2A to DNA methylation dynamics is starting to be understood. We summarize these new aspects of mechanisms to generate DNA methylomes and discuss future prospects.

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Current Opinion in Plant Biology 2022, **68**:102248

This review comes from a themed issue on **Epigenetics and gene regulation (2022)**

Edited by **Dr. Bob Schmitz** and **Dr. Ortrun Mittelsten Scheid**

For complete overview of the section, please refer the article collection - Epigenetics and gene regulation (2022)

Available online 17 June 2022

https://doi.org/10.1016/j.pbi.2022.102248

Introduction

In plant genomes, DNA methylation is enriched in transposable elements (TEs) [1,2] and the importance of DNA methylation for the control of TEs has been demonstrated using mutants defective in DNA methylation [3,4].

An important question is how DNA methylation is targeted to TEs. Control of DNA methylation has also been studied using mutants of Arabidopsis. In Arabidopsis, DNA methylation of CpG context (hereafter called mCG) is abolished in mutants defective in DNA methyltransferase (MTase) MET1 [5,6]. MET1 belongs to the maintenance DNA MTases conserved from plants to vertebrates. In addition to cytosine in CpG context, plants also methylate cytosines in other contexts, and methylation of non-CpG context (hereafter called mCH, where H can be A, T, or C) is catalyzed by a different set of DNA MTases, CHROMOMETHYLASE 2 (CMT2) and CMT3. As CMTs are recruited to regions with methylated histone H3 lysine 9 (H3K9me), mCH is abolished in mutants of H3K9 MTases, SUPPRESSOR OF VARIEGATION3-9-HOMOLOG 4 (SUVH4), SUVH5, and SUVH6. These H3K9 MTases, in turn, are recruited to regions with mCH. Thus, mCH and H3K9me are maintained by a self-reinforcing loop generated by MTases for mCH and H3K9me [7]. Both mCG and mCH//H3K9me are important for silencing TEs.

In addition to these factors necessary for "maintenance" of mCG and mCH, factors necessary for "establishment" of cytosine methylation patterns have also been investigated extensively in Arabidopsis [8,9]. Establishment of DNA methylation in Arabidopsis has been understood to rely on RNA-directed DNA Methylation (RdDM), in which small RNAs trigger the recruitment of *de novo* DNA MTases DRMs to induce both mCG and mCH. Interestingly, generation of small RNA depends on DNA methylation, forming another positive feedback loop.

Thus, the conventional view is that DNA methylation is controlled by three layers of DNA methylation pathways: maintenance of mCG by MET1, H3K9me-directed maintenance of mCH by CMTs, and small RNA-directed *de novo* mCG and mCH by DRMs; and each of these layers has the ability to be maintained independently [10]. However, recent results have revealed crosstalk between mCG and mCH that functions in both RdDM and RdDM-independent *de novo* mC pathways. Furthermore, the impact of linker histone and histone variants for the generation of DNA methylome is starting to be understood. In this review, we introduce these recent findings and discuss future challenges.

RdDM: *de novo* establishment of DNA methylation by RNAi

As insightfully summarized in multiple reviews, molecular components of RdDM have been extensively investigated in Arabidopsis [8,9,11]. Briefly, plant-specific RNA polymerase IV (Pol IV) starts to transcribe short RNA, which is used as a template for RNA-dependent RNA polymerase RDR2, and the resulting double stranded RNA is digested by DCL3 into 24-

nucleotide (nt) RNAs. Such sRNAs guide AGO4 to the long RNAs with sRNA-complementary sequences, transcribed by another RNA polymerase Pol V, and that recruits *de novo* DNA MTase DRM2 to the region of sRNA-corresponding sequence. This well-known mechanism is called canonical RdDM. Other similar mechanisms are also found and are called non-canonical RdDM [8]. RdDM of canonical and non-canonical pathways are performed by different members of the same family of each component (e.g., RDR6 vs RDR2). In addition, non-canonical RdDM pathway also uses the shorter small RNAs of 21−22-nt lengths and is currently considered as the first *de novo* mechanism for naïve DNA. But importantly, in these two types of RdDM pathways, multiple components of the machinery, including DRMs, are shared.

Crosstalk of chromatin marks in RdDM

Generally speaking, mutations of the RdDM machinery, such as *de novo* MTase DRMs, affect DNA methylation in euchromatic genomic domains; heterochromatic regions are mostly unaffected, where mCH is maintained by CH MTase CMTs. When all CH MTase genes (*DRM1*, *DRM2*, *CMT2*, and *CMT3*) are mutated to be non-functional, mCH is lost genome-wide [12]. When only *DRM*s or only *CMT*s are mutated, mCH remains in a complementary manner. The regions with remaining mCH in the *cmt2/3* mutant are called "DRM targets," because the remaining mCH should be catalyzed by DRMs. For the same reason, the regions with remaining mCH in the *drm1/2* mutant are called "CMT targets." DRM targets and CMT targets show distinct chromatin features (Table 1). Compared to CMT targets, DRM targets tend to be AT-rich, nucleosome poor, and associated with H3K4me; in short, they are euchromatic [13,14]. On the other hand, CMT targets are heterochromatic and associated with H3K9me and the linker histone H1. In addition, CMT and DRM targets have biased distribution within TEs; CMT targets are enriched in coding regions of TEs (TE genes) which are often located in internal regions of long TEs, whereas DRM targets are mainly composed of short fragments of TEs or terminal non-coding regions of long TEs [13,14] (Table 1).

Table 1

Target comparison between DRMs and CMTs.

Characteristics	DRM-targets	CMT-targets	Reference
Chromatin	Euchromatin	Heterochromatin	[12]
Target	Non-coding	Gene-coding	[13]
	Long TE (Edge)	Long TE (Internal region)	[12,14]
	Short TE		[12]
Sequence	AT-rich	GC-rich	[12,13]
Nucleosome	Poor	Rich	[12,13]
Linker histone	Poor	Rich	[14]
Histone mark	H3K4me	H3K9me H3K27me1	[12]

This table summarizes the contrasting characteristics of DRM- and CMT-targets. The references are shown on the right.

H1 is a histone protein that associates with linker regions of DNA between nucleosomes. The impact of H1 for defining RdDM targets is shown using the *h1* mutant [15]. In the *h1* mutant, RdDM affects heterochromatic regions, demonstrating the key role of H1 in excluding RdDM from heterochromatic regions. Factors binding to H3K4me and H3K9me are also involved in defining targets of RdDM. H3K4me1-binding protein RNA-DIRECTED DNA METHYLATION 15 (RDM15) is found as a component of Pol V complex and is required for sRNA biogenesis [16]. Although targets of RdDM are generally euchromatic and poor in H3K9me, a crosstalk of RdDM to H3K9me has also been found. SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1), which has H3K9me-binding activity, recruits Pol IV to the regions with H3K9me [17]. SUVH2 and SUVH9, H3K9 MTases with binding activity to mCG and mCHH, respectively, recruits Pol V to the regions with pre-existing DNA methylation, thereby accelerating RdDM [18,19]. Although the H3K9-methylation activities of SUVH2 and SUVH9 have not been detected *in vitro*, SUVH9 is necessary for H3K9me2 deposition in mature embryo, and this deposition is sRNA-dependent [20]. The relationship between RdDM and these histone modifications, as well as H1, is still enigmatic and offers exciting future research materials.

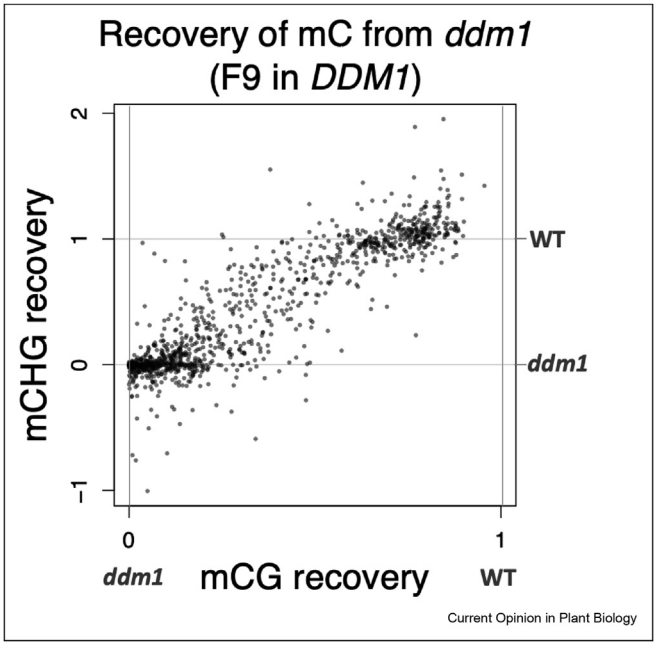
Although CMT targets keep mCH in *drm1 drm2* mutant background, it is possible that mCH in CMT targets are initially established by RdDM and thereafter maintained by CMTs. Consistent with this scenario, it is recently proposed that RDR6-mediated RdDM can be induced by ribosome stalling of TE genes with unoptimized codon usage [21]. Importantly, because the RNAs undergoing translation should correspond to coding regions, this mechanism could account for the initial step of RdDM in the CMT targets. Although it remains to be seen how general this intriguing pathway is, this proposal is consistent with the facts that transcription by Pol II is required at the first stage of RdDM [8,22], and that Pol II-derived siRNAs can guide AGO4-clade ARGONAUTE proteins to localize Pol V to the unmethylated DNA [23,24]. Another interesting observation in regard to the trigger of RdDM is that evolutionarily old TEs within the genome of Arabidopsis and tomato tend to be targeted by RdDM more than young TEs [25]. This effect can be due to frequent base substitutions of methylated C (mC) to T, which makes the TE gene sequences AT-rich and targets of RdDM.

RdDM-independent CH methylation establishment (RiCHE)

We discussed the possibility that RdDM is involved in the establishment of DNA methylation even at the CMT targets. To characterize such activity genome-wide, a powerful approach would be to follow *de novo* DNA methylation after loss and reintroduction of factors necessary for DNA methylation.

DECREASE IN DNA METHYLATION 1 (DDM1) is a chromatin remodeling factor involved in heterochromatin maintenance; in *ddm1* mutants, mCG, mCH, and H3K9me are all lost in the CMT targets [13,26]. The loss of DNA methylation in *ddm1* does not recover efficiently even after genetic reintroduction of the *DDM1* wild-type allele; the *ddm1*-induced hypomethylation remains over multiple plant generations in the *DDM1* background [27,28]. The *ddm1*-induced loss of H3K9me is also heritable to the progeny [29]. Slow recovery of the DNA methylome is detected in a subset of TE genes, which becomes evident after multiple plant generations [30]. Interestingly, this slow recovery occurs simultaneously in mCG and mCH in each TE gene (Figure 1).

Figure 1



mCG and mCH lost in a *ddm1* mutant recover simultaneously. The *ddm1* mutant loses both mCG and mCH in TEs. The recovery of DNA methylation was examined in the progeny of *ddm1* after genetic introduction of a functional *DDM1* allele. The *ddm1*-derived hypomethylated genomic regions generally remain hypomethylated even in the wild-type *DDM1* background [27,28]. After multiple generations in the *DDM1* background, however, slow but consistent recovery was detected in a subset TEs with small RNAs [30]. Interestingly, mCG and mCH recover simultaneously [14]. Each dot represents the relative recovery level of mCG and mCHG compared to *ddm1* and WT in the coding region of TE that locates within *ddm1*-derived genomic regions in the F9 generation (the 9th generation in the *DDM1* wild-type background). The recovery levels are calculated by (F9 − *ddm1*)/(WT − *ddm1*). The recovery in mCHH also correlates with that in mCG (not shown). Original data from Colomé-Tatché et al. [68]. This figure is modified and reproduced from To et al., 2020 (Extended Data Fig. 3c) [14].

Therefore, key experiments would be to observe recovery of mCG and mCH separately after the loss of only one of them. Global loss of mCG in mutant of CG MTase MET1 is inherited to the progeny [6,31,32], as is the case for the *ddm1*-induced loss of heterochromatin marks. Importantly, for both *ddm1*-induced and *met1*-induced loss of DNA methylation, the recovery depends on RdDM [14,30,32,33]. On the other hand, analogous experiments done for mCH show very different and unexpected results.

In the *cmt2/3* double and the *suvh4/5/6* triple mutants, both mCH and H3K9me are lost in the CMT targets. In contrast to the case for *ddm1*, the genetic reintroduction of functional genes immediately induces *de novo* establishment of both mCH and H3K9me [14]. Importantly, this establishment of heterochromatin marks is RdDM-independent; these marks can be established *de novo* in the backgrounds without functional RdDM machinery. We refer to this pathway as RdDM-independent CH methylation Establishment, or RiCHE in this review. As RiCHE occurs in the absence of DRM2 [14], CMTs are suggested to act as *de novo* MTases instead. This interpretation is consistent with the previous results suggesting *de novo* MTase activity of CMT3 *in vitro* [34] and *in vivo* [35,36]. As TE genes lose both mCH and H3K9me in *cmt2/3* or *suvh4/5/6* mutant background, an important next question is how hosts identify TE genes to re-introduce mCH and H3K9me in the RdDM-independent pathway. Recent results by multiple groups suggest that mCG is important for identifying the targets of RiCHE (Figure 2), as discussed in the next section.

Crosstalk between mCG and mCH pathways

Both mCG and mCH are enriched in TEs, but they are almost independently maintained. Recent studies have revealed their crosstalk for *de novo* establishment of mCG and mCH [14,32,37,38]. The crosstalk is seen for both RdDM-dependent and -independent pathways.

Although maintenance of mCG is largely independent of mCH, a small subset of TE genes loses mCG when mCH and H3K9me are lost in the *cmt2/3* or *suvh4/5/6* mutant backgrounds. Importantly, RiCHE does not function efficiently in these TE genes with reduced mCG, suggesting the importance of mCG for RiCHE [14]. Furthermore, RiCHE does not function when mCG is lost in a *met1* mutant background, demonstrating that mCG is necessary for RiCHE [38]. The

dependence of RiCHE on mCG is also consistent with the results from the *ddm1* mutant; the mCH recovery from *ddm1* is much less efficient, as it also loses mCG.

mCG also guides mCH to euchromatic non-TE genes [37,38], although this effect is normally masked by the H3K9 demethylase IBM1 (INCREASE IN BONSAI METHYLATION 1), which excludes mCH/H3K9me from active genes [39]. It is tempting to speculate that mCH induction in the *ibm1* mutants share mechanisms with RiCHE, as both depend on mCG and both are independent of RdDM [14,37,38,40].

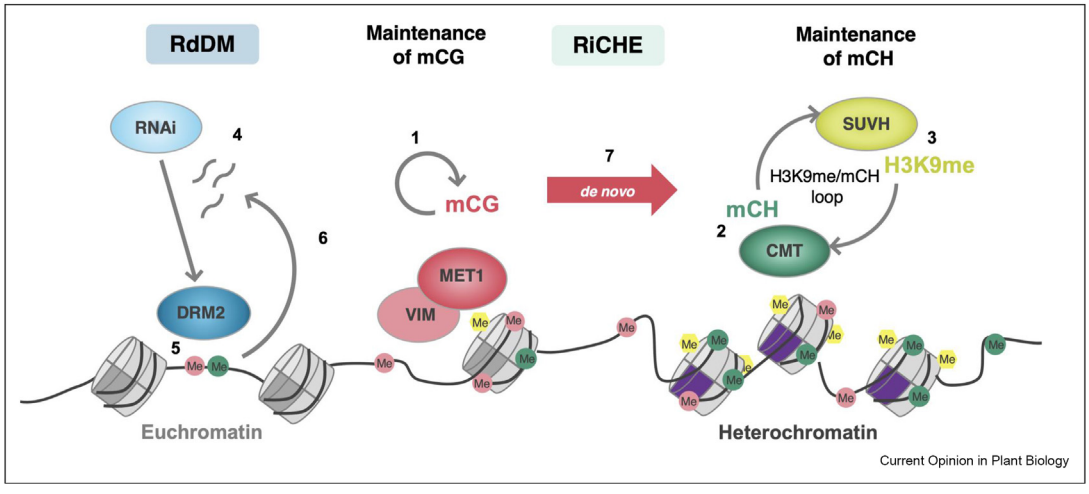
The opposite direction of reinforcement, mCH/H3K9me to mCG, is also observed, although in that case, it is mediated by RdDM. As briefly discussed in the previous section, RdDM-dependent recovery of mCG is detected after its loss in both *ddm1* and *met1* mutant. Interestingly, the recovery is much less efficient from *ddm1* than from *met1* [14,30,32]. The mCG recovery from *met1* is especially enhanced in TE genes with mCH and H3K9me, further suggesting that mCH enhances RdDM of mCG.

Thus, although mCG and mCH/H3K9me are mostly maintained independently, they reinforce each other during their establishment, accounting for the synergistic colocalization of mCG and mCH/H3K9me to silence TEs. The crosstalk between mCG and mCH also accounts for the extremely inefficient recovery of mCH and mCG after their loss in the *ddm1* mutant. Simultaneous loss of these marks makes the recovery inefficient.

In addition to these effects on mC and H3K9me, the *ddm1* mutation affects accumulation of the histone variant H2A.W in the heterochromatic TEs [41]. H2A.W localizes in heterochromatin [42] and H2A.W interacts with the linker DNA [43]. Interestingly, H2A.W competes with H1 to inhibit excessive H1 incorporation in heterochromatin and affects chromatin accessibility and DNA methylation [44].

While H2A.W is an epigenetic mark of silent TEs, another H2A variant, H2A.Z, is found in active or inducible genes. Interestingly, H2A.Z and mCG distribute in mutually exclusive manner in species ranging from Arabidopsis to puffer fish [45,46]. In Arabidopsis, H2A.Z can act as a recruiter of REPRESSOR OF SILENCING 1 (ROS1), which catalyzes DNA demethylation [47]. Interestingly, replacement of H2A variants is associated with efficiency of RiCHE [14], and H2A variants could possibly play a role in the crosstalk between mCG and mCH.

Figure 2



Two modes of mCH establishment. This figure summarizes maintenance and establishment of mCG and mCH. mCG is maintained by MET1 with its recruiter proteins VARIANT IN METHYLATION (VIM) (1). mCH is maintained by self-reinforcing loop with H3K9me; mCH MTases CMT2 and CMT3 is directed to heterochromatic regions with H3K9me (2), while H3K9 MTases SUVH4, SUVH5, and SUVH6 are recruited to regions with mCH (3), resulting in maintenance of these marks. mCG and mCH can be established *de novo* by RNAi-dependent and -independent pathways, shown as RdDM and RiCHE in this figure. RNAi-dependent DNA methylation mechanism, called RNA-directed DNA Methylation (RdDM), is very well characterized, although rather simplified in this figure. The first step of RdDM is generation of small RNAs (4), and the final step of canonical and non-canonical RdDM pathways [8], is *de novo* mCG and mCH by DNA MTase DRM2 (5). Methylated cytosines can feedback to siRNA biogenesis through mC-binding SUVH proteins, SUVH2 and SUVH9 (6). mCH can also be established by an RNAi-independent pathway, named RiCHE. Components of RdDM, such as DRM2 and RDRs are dispensable for this process. RiCHE depends on mCG (7), but the molecule(s) mediating this crosstalk remains to be investigated. Although mCG (1) and mCH/H3K9me (2,3) are mostly independent with regard to the maintenance, their crosstalk during establishment is detected (7).

Regarding the mode of interaction between mCG and mCH, recent report on their dynamics during cell cycle progression is suggestive. mCG is rapidly duplicated (by G2 phase) to the newly synthesized chromosome, while mCH is acquired later [48]. This transition might ensure accurate maintenance of mCH by pre-existing mCG.

During RdDM and RiCHE, local crosstalk between mCG and mCH/H3K9me can stabilize and enhance each other, to ensure robust silencing of TEs. However, as positive feedback also carries a risk of over-amplification, plants appear to have mechanisms to negatively regulate heterochromatin marks by sensing a global mCG levels [37,38,49]. Induction of mCH by mCG in TE genes and active genes is enhanced when global mCG level is low. Global loss of mCG induced in *ddm1* and *met1* is associated with ectopic CH methylation in CG-methylated genic regions or heterochromatic regions [49–51]. In addition, mCH accumulation in the *ibm1* mutant is associated with a decrease in mCH at heterochromatic TEs [38]. These observations suggest that plants have mechanisms to balance global mCH levels.

Analogous observations in other organisms

The significance of mCH for gene silencing is also suggested in *Physcomitrella patens*. While mCH is less predominant than mCG in Arabidopsis, it is as abundant as mCG in *Physcomitrella*, and its loss causes transcriptional derepression of TEs more severely than the loss of mCG [52]. Interestingly, *PpCMT*, the *Physcomitrella* ortholog of CMT, seems to have *de novo* CHG MTase activity, which is independent of RNAi [53].

mCH is also found in mammals, for examples, in neural cells and stem cells, and its dysfunction results in developmental abnormalities in neural and iPS cells [54–57].

The crosstalk between mCG and H3K9me is also observed in mammals. The *de novo* DNA methylation activity by DNMT1, the CpG maintenance MTase orthologous to MET1, has recently reported and this activity is enhanced by neighboring H3K9me2/3 [58]. DNMT1 binds to Ub/K9me3 bi-modified H3, and the presence of H3K9me3 can directly enhance mCG [59]. In addition, mouse H3K9me2 MTase EHMT2 directs DNA methylation [60].

Crosstalk with transcription

As discussed above, active and inactive epigenome patterns are stabilized by multiple positive feedback loops. The feedback can also involve transcription. The H3K9 demethylase IBM1 removes H3K9me from transcribed genes, which further ensures active transcription [39,61]. It would be interesting to learn whether other factors downstream of transcription also affect epigenome.

Genetic screening of factors mediating gene silencing caused by ectopic genic H3K9me/mCH in the *ibm1* mutant identified LSD1-LIKE 2 (LDL2), which encodes a demethylase for H3K4me1 [62]. Thus, loss of H3K4me1 may mediate silencing by mCH/H3K9me. H3K4me1 can be controlled in both upstream and downstream of transcription [63,64] and could play a role in stabilization of the epigenetic states.

In many organisms including animals and fission yeast, silencing by H3K9me is mediated by a heterochromatin protein, HP1. Arabidopsis LIKE HETEROCHROMATIN PROTEIN1 (LHP1) is regarded as the HP1 ortholog, but LHP1 binds primarily to H3K27me3, rather than H3K9me. Instead of HP1, an unrelated protein AGDP1 (AGENET DOMAIN (AGD)-CONTAINING P1)/ADCP1 (AGENET DOMAIN CONTAINING PROTEIN 1) has functions comparable to those of HP1 [65,66]. This protein binds to H3K9me and induces heterochromatin condensation and TE silencing. In addition, this protein is necessary for maintaining H3K9me2 and mCH, possibly reflecting positive feedback from downstream events.

In mammals, mCG is recognized by methyl CpG binding domain proteins (MBD) and induces downstream silencing pathways. Similarly, Arabidopsis MBD5 and MBD6 bind to mCG and their loss-of-function mutations induce derepression of a subset of transcription units silenced by mCG [67]. Furthermore, an exploration of interacting proteins with MBD5 and MBD6 identified a J-domain protein SILENZIO as a silencing effector, which also binds HSP70 chaperones and recruits them, through the methyl-targeting activity of MBD5/6, to the DNA methylated sites.

Conclusion and perspectives

The combination of genetics and epigenomics has been powerful to detect the crosstalk of modifications, not only for their maintenance, but also for their establishment. Although the frameworks for multiple important pathways have been detected using genetic approaches, many of the molecular components remain to be understood. The black boxes include factors mediating the crosstalk between mCG and mCH during their establishment, factors downstream of mCG and mCH, and the effects of histone variants to the dynamics of modifications of histones and DNA. Further genetic and genomic studies on Arabidopsis will be effective and, when combined with structural and biochemical approaches, open new perspectives for understanding plant epigenomes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Elizabeth Dennis, Eric Richards, and Robert Schmitz for comments on the manuscript. This work was supported by grants from the Human Frontier Science Program (RGP0025/2021 to T.K.) and the Japanese Ministry of Education, Culture, Sports, Science and Technology (22K06180, 19H05740 and 17K15059 to T.K.T., 19H00995 and 21H04977 to T.K.).

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