PERSPECTIVE

Seeing the forest for the trees: a wide perspective on RNA-directed DNA methylation

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In this issue of *Genes & Development*, Wierzbicki and colleagues (pp. 1825–1836) examine the current model of RNA-directed DNA methylation (RdDM) by determining genome-wide distributions of RNA polymerase V (Pol V) occupancy, siRNAs, and DNA methylation. Their data support the key role of base-pairing between Pol V transcripts and siRNAs in targeting de novo DNA methylation. Importantly, the study also reveals unexpected complexity and provides a global view of the RdDM pathway.

DNA methylation at the fifth position of cytosine regulates many critical biological processes, such as gene imprinting, silencing of transposable elements, and X-chromosome inactivation. In *Arabidopsis*, the DNA methyltransferase DRM2 (domains rearranged methyltransferase 2) catalyzes de novo methylation in all cytosine contexts, including CG, CHG, and CHH (H represents either A, T, or G) (Cao and Jacobsen 2002). The targeting of DRM2 for DNA methylation can be achieved by the RNA-directed DNA methylation (RdDM) pathway that consists of three phases: biogenesis of 24-nucleotide (nt) siRNAs, production of scaffold RNAs, and recruitment of DRM2 assisted by complementary pairing between 24-nt siRNAs and nascent scaffold RNAs.

RNA polymerases in the RdDM pathway

In the RdDM pathway, transcription of the noncoding RNAs involves three DNA-dependent RNA polymerases: Pol II, Pol IV, and Pol V. *Arabidopsis* Pol IV and Pol V are plant-specific RNA polymerases, each of which contains 12 subunits (Ream et al. 2009). While six of these 12 subunits are identical among Pol IV, Pol V, and Pol II, the

remaining subunits of Pol IV and/or Pol V are different but have apparently evolved from their paralogs in Pol II (Ream et al. 2009). NRPD1 and NRPE1 are the unique and largest subunits of Pol IV and Pol V, respectively. Mutation in NRPD1 or NRPD/E2, which is the second largest subunit shared by Pol IV and Pol V, resulted in >90% reduction of 24-nt siRNA accumulation (Zhang et al. 2007; Mosher et al. 2008), thereby demonstrating a predominant role of Pol IV in generating 24-nt siRNAs. Although Pol IV transcripts remain to be identified, Pol IV has been thought to initiate siRNA production by generating an aberrant ssRNA, which is subsequently copied by RDR2 (RNA-dependent RNA polymerase 2) to produce dsRNAs that are cleaved by DCL3 (dicer-like 3) and then loaded onto AGO4 (argonaute 4) or its closely related argonaute proteins (Law and Jacobsen 2010; Haag and Pikaard 2011; Zhang and Zhu 2011). These argonaute-bound 24-nt siRNAs can then serve as sequence-specific guides for methylation by pairing with complementary DNA or nascent scaffold RNA. AGO4 can be cross-linked to scaffold RNAs, supporting the model of siRNA-scaffold RNA pairing (Wierzbicki et al. 2009).

Production of scaffold RNA is independent of siRNA biogenesis, as shown in *Arabidopsis* mutants defective in NRPD1, RDR2, or DCL3 (Wierzbicki et al. 2008). Wierzbicki et al. (2008) previously demonstrated that Pol V is necessary for scaffold RNA production. In addition to generating scaffold RNAs, Pol V can reinforce siRNA production at some RdDM target loci (Zhang et al. 2007; Mosher et al. 2008) and can also interact with AGO4 through an argonaute-binding motif in the C-terminal tail of NPRI (El-Shami et al. 2007), thereby strengthening the recruitment of the silencing complex guided by AGO4. Direct interaction between AGO4 and the methyltransferase DRM2 has not been shown. Nevertheless, cytological analyses revealed colocalization of AGO4 and DRM2, together with other RdDM components in distinct nuclear foci (Li et al. 2006, 2008; Pontes et al. 2006; He et al. 2009; Gao et al. 2010). Additionally, AGO4 and DRM2 both coimmunoprecipitate with RDM1 (RNA-directed DNA methylation 1), which is an ssDNA-binding protein with preference to methylated DNA (Gao et al.

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2010). Therefore, Pol V and its transcriptional products seemed to be essential for targeting de novo DNA methylation.

Transcription for noncoding RNAs during RdDM can also be Pol II-dependent. Pol II transcription of inverted DNA repeats can generate dsRNAs that serve as DCL3 substrates, thus contributing to 24-nt siRNA production (Zhang et al. 2007; Pikaard et al. 2008). At some intergenic low-copy-number repeat loci, Pol II was shown to be indispensable for scaffold RNA generation (Zheng et al. 2009). Like Pol V, Pol II also possesses the GW/WG repeats that bind to AGO4 (Zheng et al. 2009). Although the argonaute-binding motif appears to be weaker in Pol II than Pol V, Pol II clearly interacts with AGO4 and RDM1 (Zheng et al. 2009; Gao et al. 2010). In a weak loss-of-function allele of the NRPE2 (the second largest subunit of Pol II) mutant, occupancy of Pol IV and Pol V at some heterochromatic loci is reduced (Zheng et al. 2009), indicating that these three RNA polymerases may cooperate during RdDM. Meanwhile, NRPE1 colocalizes with RDM1, DRM2, and AGO4 in the perinucleolar bodies but shows no clear colocalization patterns in nucleoplasmic foci (Li et al. 2006, 2008; Pontes et al. 2006; He et al. 2009; Gao et al. 2010). In contrast, colocalization between NRPE1 and RDM1 or AGO4 can only be observed in discrete nucleoplasmic foci (Gao et al. 2010). Therefore, it appears that Pol V and Pol II can mediate RdDM in a spatially separated manner. It is unclear whether the activities in the perinucleolar bodies and nucleoplasmic foci may be parallel or sequential (Zhang and Zhu 2011).

Genome-wide examination of the RdDM model

Although a key role of Pol V in RdDM has been established, only a few scaffold RNAs had been detected before the study by Wierzbicki et al. (2012). In addition, Pol V also mediates RdDM-independent silencing at pericentromeric repeats (Douet et al. 2009; Pontes et al. 2009). It was unknown how broadly Pol V is needed across the whole genome. By using ChIP-seq (chromatin immunoprecipitation [ChIP] followed by deep sequencing), Wierzbicki et al. (2012) compared genome-wide NRPE1 occupancy in wild-type *Arabidopsis* and the *nrpe1-11*-null mutant and thereby revealed potential Pol V-transcribed sites throughout the whole genome. NRPE1 occupies >1000 genomic regions, including loci that had been known to be transcribed by Pol V. These Pol V-occupied regions range from several hundred to a few thousands of base pairs and are distributed throughout each chromosome except the centromeric regions. Similarly, mutation of NRPE1 tends to reduce siRNA accumulation at loci that are dispersed across the genome rather than enriched at the centromeric regions, as revealed by the same study and another recent report (Lee et al. 2012).

Prior to the study by Wierzbicki et al. (2012), the RdDM model had been examined at only a small number of heterochromatic loci, where production of both 24-nt siRNAs and nascent scaffold RNAs was assumed to be a prerequisite for de novo DNA methylation. To test

the RdDM model genome-wide, Wierzbicki et al. (2012) also performed parallel analyses of the DNA methylome and siRNA transcriptome. Among the identified Pol V-occupied loci, a majority (56%) encompasses regions where complementary 24-nt siRNAs and the asymmetric CHH methylation can be found. Such observations support the RdDM model in which the pairing between siRNA and scaffold RNA recruits the silencing complex, given that methylation in CHH context must be maintained by de novo methylation and is therefore a hallmark of RdDM activity. In contrast, only 1% of Pol V target sites show CHH methylation without the 24-nt siRNA.

Locus-specific reduction of DNA methylation has been well documented in *Arabidopsis* mutants with defective RdDM. Wierzbicki et al. (2012) observed an unexpected shift, instead of a global loss, of the CHH methylation mark in Pol IV and Pol V mutants compared with wild-type plants. In the mutants, ~50% of CHH cytosine became unmethylated, and this was accompanied by the ectopic occurrence of a similar number of CHH methylations at new positions. Intriguingly, CHH methylation was reduced in the chromosome arms, while new CHH methylation occurred in the pericentromeric regions. These results indicate that both Pol IV and Pol V function in targeting DNA methylation to certain loci but are not required for DNA methyltransferase activities, and in the absence of these polymerases, DNA methylation is redirected to other loci.

Discovered and to be discovered

The discoveries made by Wierzbicki et al. (2012) have allowed a genome-wide evaluation of the role of noncoding RNA pairing in mediating de novo DNA methylation. In addition to providing support for the current RdDM model, their results also provide novel information that will cause researchers to evaluate de novo DNA methylation from a broader perspective (Fig. 1).

In *nrpd1*, *nrpe1*, and *nrpd/e2* mutants, a significant portion (slightly <50%) of cytosine positions in the CHH context remain to be methylated (Wierzbicki et al. 2012). One possible explanation is that RNA-directed de novo DNA methylation at these positions is at least in part independent of Pol IV and Pol V. Generation of 24-nt siRNAs and scaffold RNAs at these loci may also be accomplished by Pol II, which is known to be involved in RdDM in addition to having a canonical role in mRNA transcription (Zheng et al. 2009). Similarly, in mammals (which lack both Pol IV and Pol V), piRNAs (25- to 30-nt small RNAs known to mediate post-transcriptional silencing of transposons) have been suggested to pair with nascent transcripts and recruit de novo DNA methyltransferases (Watanabe et al. 2011). It would be interesting to determine whether these persistent CHH methylation marks can be abolished or significantly reduced by triple mutations of the three RNA polymerases.

Alternatively, persistence of CHH methylation in Pol IV and Pol V mutants may indicate the existence of other factors that can direct de novo DNA methylation in-

Figure 1. A genome-wide view of Pol V functions and de novo DNA methylation in *Arabidopsis*. For the chromosome depicted in the center of the figure, black and yellow indicate the centromeric and pericentromeric regions, respectively. Red bars on the chromosome show regions of interest, as illustrated in A–F. (A,B) Some Pol V-occupied loci exhibit neither 24-nt siRNA production nor any cytosine methylation (A), while most overlap sites of both 24-nt siRNA production and cytosine methylation in the CHH context (^mCHH), which are indicative of RdDM (B). (C) Pol V also associates with DNA sequences that contain cytosine methylation other than ^mCHH, possibly contributing to RdDM-independent silencing. In the *nrpd1*, *nrpe1*, and *nrpd/e2* mutants, positions of cytosine methylation in the CHH context can be lost, gained, or retained relative to the wild type (WT), as indicated. (D) Loss of ^mCHH can be attributed to dysfunction of the Pol IV- and Pol V-dependent RdDM pathway. (E) Gain or retention of ^mCHH in the mutants may be mediated through Pol II-dependent RdDM and/or DNA methylation that is recruited simply via protein interactions. See the text for additional information.

independently of noncoding RNAs. It is unclear whether regions enriched with persistent CHH methylation are devoid of 24-nt siRNA production and/or Pol V occupancy. DNA methyltransferases such as MET1, which can be recruited by protein interactions during DNA replication (Law and Jacobsen 2010), can potentially catalyze CHH methylation in the absence of the RdDM pathway, as indicated by the *drm1 drm2 cmt3* triple mutant that retains a substantial level of CHH methylation (Cokus et al. 2008). Protein interactions might also enable recruitment of DRM2 without the assistance of pairing between 24-nt siRNAs and scaffold RNAs. DRM2 physically associates with RDM1 (Gao et al. 2010), one of the components of the DDR complex that also contains the putative chromatin-remodeling protein DRD1 (defective in RdDM 1) and the chromosome hinge domain protein DMS3 (defective meristem silencing 3) (Law et al. 2010). Although the DDR complex is necessary for Pol V transcription and the RdDM pathway (Law et al. 2010), it is possible that this protein complex alone or other protein complexes are sufficient to recruit DRM2.

Pericentromeric regions are enriched with repetitive sequences and are heavily methylated (Copenhaver et al. 1999; Zhang et al. 2006). In the absence of Pol IV and/or Pol V, pericentromeric regions gain thousands of new, ectopic sites of CHH methylation (Wierzbicki et al. 2012). If such methylation is catalyzed by DRM2, these results would seem to indicate a competition for methylation targeting. In other words, DRM2 is normally recruited by Pol IV- and Pol V-dependent noncoding RNA pairing to chromosome arms in wild-type plants. However, in the absence of these polymerases, DRM2 would be targeted to pericentromeric regions, possibly aided by Pol II-dependent noncoding RNAs or other factors. The abundant DNA methylation in pericentromeric regions requires CMT3 and MET1 to maintain cytosine methylation in the CHG and CG contexts, respectively (Law and Jacobsen 2010). CMT3 and MET1 may also catalyze de novo methylation (Cao et al. 2003; Aufsatz et al. 2004). However, a significant occurrence of newly methylated cytosine positions was observed only in the CHH context (Wierzbicki et al. 2012). Mutation of NRPD1 or NPRPE1 in *Arabidopsis* leads to repression of

gene expression of ROS1 (Huettel et al. 2006), which is a major DNA demethylase that erases or prunes DNA methylation at thousands of loci (Qian et al. 2012). Therefore, impaired active demethylation may also contribute to the ectopic occurrence of CHH methylation at new positions.

The results by Wierzbicki et al. (2012) clearly support the idea that Pol IV- and Pol V-dependent siRNA-scaffold RNA pairing guides de novo methylation. In an independent study, Zhong et al. (2012) also observed that, on a whole-genome scale, Pol V targets were tightly correlated with DNA methylation and small RNA accumulation. Still, what determines Pol V occupancy throughout the whole genome remains unclear. Two multinucleotide motifs were identified in 50% of the Pol V-occupied loci (Wierzbicki et al. 2012). These motifs, however, cannot explain Pol V recruitment because >65,000 such motifs are present across the chromosomes, with rare concordance with sites of Pol V enrichment. The Zhong et al. (2012) study suggested that Pol V has a tendency to associate with gene promoters, and the association becomes stable when the promoters contain transposons. Zhong et al. (2012) also found that ~80% of the Pol V-targeted transposons are evolutionarily young, indicating that Pol V has evolved to mediate RdDM for genome surveillance. Interestingly, Pol V association with transposons is clearly characterized by its enrichment at transposon edges (Zhong et al. 2012). Production of scaffold RNAs during RdDM requires DRD1 (Wierzbicki et al. 2008; Zheng et al. 2009), and meanwhile, the DDR complex physically interacts with Pol V (Gao et al. 2010; Law et al. 2010). In fact, global chromatin association of Pol V is dependent on the DDR complex (Zhong et al. 2012). Thus, one possibility is that chromatin remodeling by DDR might precede Pol V occupancy for RdDM. Wierzbicki et al. (2012) also found that Pol V occupancy is not always tied to heterochromatin. Among 1157 Pol V-occupied sequences, 27% show neither complementary 24-nt siRNAs nor cytosine methylation in any context, and more than half of the Pol V-occupied loci overlap genes. Similar to these euchromatin-associated patterns, Pol V seems to affect small RNA production mainly from intergenic regions in euchromatin (Lee et al. 2012). The frequent presence of Pol V in euchromatin suggests novel, yet-to-be-explored roles.

Mutation of Pol V causes depletion of the repressive histone H3 Lys 9 dimethylation (H3K9me₂) mark and decondensation of pericentromeric chromocenters, a process independent of Pol IV, RDR2, DCL3, AGO4, or DRM2 (Pontes et al. 2009). Of the Pol V-occupied regions, 8% exhibited 24-nt siRNA production but not CHH methylation (Wierzbicki et al. 2012). It is possible that active DNA demethylation counteracts RdDM at some of these loci and thereby masks cytosine methylation. Alternatively, this pattern may reaffirm a role of Pol V in mediating RdDM-independent silencing because this type of loci includes transposons and pericentromeric repeats (Wierzbicki et al. 2012). The DDR complex is required for Pol V to associate with chromatin (Zhong et al. 2012). Mutation of DRD1 has effects similar to

nrpe1 in heterochromatin decondensation (Pontes et al. 2009), and bacterially expressed AtDMS3 interacts with AtMORC6, a MORC ATPase whose mutation causes decondensation of pericentromeric heterochromatin (Lorković et al. 2012; Moissiard et al. 2012). Therefore, physical association with chromatin remodeling proteins likely contributes to Pol V occupancy in pericentromeric regions.

Conclusion

Comparative analyses of Pol V occupancy, the DNA methylome, and the siRNA transcriptome have provided strong support for the current RdDM model from a whole-genome perspective. Investigations of global DNA methylation patterns also revealed complexities that were not detected in earlier locus-specific studies. Along with previous findings, these important discoveries depict a genome-wide outlook of Pol V functions as well as of de novo DNA methylation. Further work that addresses new questions raised in these studies will lead to a better understanding of Pol V functions and RdDM.

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