OUTLOOK

Broken up but still living together: how ARGONAUTE's retention of cleaved fragments explains its role during chromatin modification

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Throughout the eukaryotic kingdoms, small RNAs direct chromatin modification. ARGONAUTE proteins sit at the nexus of this process, linking the small RNA information to the programming of chromatin. ARGONAUTE proteins physically incorporate the small RNAs as guides to target specific regions of the genome. In this issue of *Genes & Development*, Wang and colleagues (pp. XXX–XXX) add substantial new detail to the processes of ARGONAUTE RNA loading, preference, cleavage, and retention, which together accomplish RNA-directed chromatin modification. They show that after catalytic cleavage by the plant ARGONAUTE protein AGO4, the cleaved fragment remains bound. This happens during two distinct RNA cleavage reactions performed by AGO4: first for a passenger RNA strand of the siRNA duplex, and second for a nascent transcript at the target DNA locus. Cleaved fragment retention of the nascent transcript explains how the protein complex accumulates to high levels at the target locus, amplifying chromatin modification.

In order to silence the expression of transposable elements and other regions of the genome, eukaryotic cells use three methods of targeting new repressive chromatin modifications. First, the presence of pre-existing chromatin modifications can direct the recruitment of additional rounds of modification. Second, transcription factor proteins can directly bind specific DNA sequences and direct chromatin modification. Third, chromatin modifications can be targeted through the action of small RNAs. In protists, plants, fungi, and animals, the action of ARGONAUTE (AGO) proteins is programmed by the incorporation of a small RNA molecule (for review, see Höck and Meister 2008). This process is essential for localizing AGOs to chromatin in order to initiate chromatin modification (Shimada et al. 2016), even though the types of small RNAs and their biogenesis can differ between species.

In the model organism *Arabidopsis thaliana*, the mechanism of RNA-directed DNA methylation (RdDM) has been dissected (for review, see Zhang et al. 2018). Since plants do not have transcription factor-based silencing of transposable elements, RdDM plays a central role in initiating transposable element silencing and maintaining robust chromatin boundaries between euchromatic genes and neighboring heterochromatic transposable elements (Li et al. 2015; Sigman et al. 2021).

A key protein in plants responsible for RdDM is AGO4. AGO4 is programmed by 24-nt small interfering RNAs (siRNAs), which direct base pairing with a nascent transcript that is still attached to the DNA locus. AGO4 incorporates a duplex of double-stranded siRNA in the cytoplasm after export of the siRNA duplex from the nucleus (Fig. 1A; Ye et al. 2012). The double-stranded RNA duplex has both a guide strand, which will guide AGO4 to complementary sequences, and a passenger strand, which will be cleaved and not used. After loading, AGO4 slices the passenger strand (Fig. 1B) and is imported into the nucleus (Fig. 1C) to direct RdDM (Fig. 1D). Although AGO4 has been extensively studied, key questions remained. For example, how it is selected which RNA strand is the passenger to be cleaved versus the guide strand to target AGO4.

In this issue of *Genes & Development*, Wang et al. (2023) used in vivo AGO4-RNA immunoprecipitation paired with sequencing alongside the creation of an in vitro AGO4 loading and cleavage assay to generate a deeper understanding of AGO4 action. They found that even after AGO4 catalytic activity and slicing, AGO4 retains binding of the cleaved fragment. AGO4 performs two

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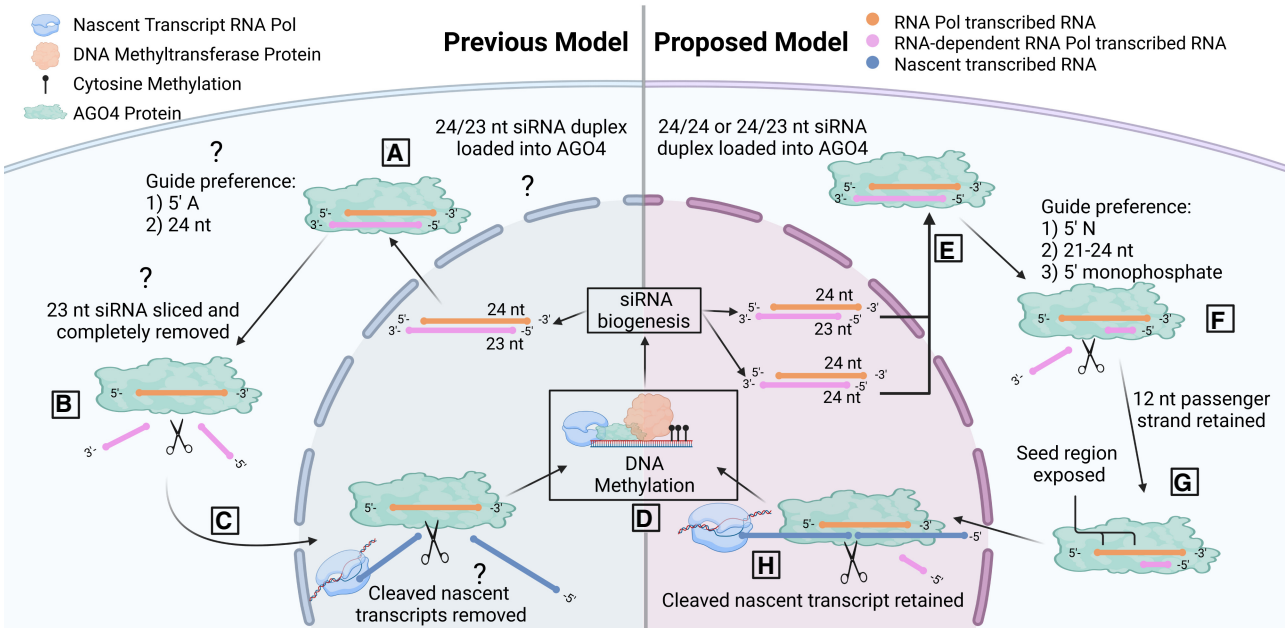

Figure 1. AGO4 mechanism during RdDM. (*Left*) Previous model, in which 24-/23-nt siRNA duplexes are loaded into AGO4. (A) AGO4 chooses the 24-nt strand that has a 5′ A on the guide strand. (B) The 23-nt strand is cleaved and eliminated. (C) AGO4 is imported into the nucleus and cleaves a nascent transcript at the DNA locus, resulting in (D) RNA-directed DNA methylation. (*Right*) New proposed model. (E) Both 24-/23- and 24-/24-nt siRNA duplexes are loaded into AGO4. (F) In the 24/23 duplex, the 24-nt strand is recognized as the guide. In the 24/24 duplex, the strand that contains a 5′ monophosphate is recognized as the guide. (G) AGO4 cleaves the passenger strand and retains the 5′ 12-nt fragment. (H) AGO4 is imported into the nucleus, where it cleaves a nascent transcript, retaining the cleaved fragments and remaining at the DNA locus for (D) RNA-directed DNA methylation. (Created with BioRender.com.)

separate cleavage reactions during RdDM. Retention of the cleaved fragment was found with both the passenger RNA strand just after AGO4 loading of the duplex siRNA in the cytoplasm (Fig. 1G) and the nascent RNA transcript after cleavage in the nucleus (Fig. 1H).

Diving deeper, the first key finding by Wang et al. (2023) is that AGO4 can incorporate duplexes of 24/24 nt or 24/23 nt (Fig. 1E). In the case of the 24-/23-nt duplex, the 23-nt strand serves as the passenger and is cleaved, generating a 12-nt fragment that remains associated with AGO4. For 24-/24-nt duplexes, one strand is recognized as the guide strand, likely by the 5′ monophosphate on the strand made by an RNA polymerase rather than the 5′ triphosphate on the strand generated by an RNA-dependent RNA polymerase protein (Fig. 1F; Singh et al. 2019).

Equally important, Wang et al. (2023) found that in vitro AGO4 does not have the strict preference for 24-nt siRNAs or the 5′ adenosine base characteristic of AGO4-bound siRNAs in vivo (Mi et al. 2008; Havecker et al. 2010). Rather, these in vivo observations are the result of cleavage and biogenesis preferences of siRNAs by proteins upstream of AGO4 (Loffer et al. 2022). This is important because previous work has shown that the closely related protein AGO6 is able to incorporate shorter 21- to 22-nt siRNAs and trigger the establishment of RdDM using siRNAs generated by other pathways (McCue et al. 2015). Together, these works demonstrate that AGO4 and related proteins are more flexible in their RNA binding capacities than previously thought.

Once translocated into the nucleus, Wang et al. (2023) now suggest that the seed region of the guide RNA strand, which is exposed due the removal of the passenger strand 11- to 12-nt cleavage fragment, can initiate base pairing to the nascent transcript (see Fig. 1H). They demonstrate the first direct biochemical evidence of slicing of nascent transcripts by AGO4. This function had been previously inferred from patterns of in vivo sequencing data (Liu et al. 2018) and genetic evidence of loss of some RdDM in catalytically inactive mutant versions of AGO4 (Qi et al. 2006).

AGO4 localization at the target locus is important, and the previous model suggested that a lack of cleavage would retain AGO4 at this locus longer than if AGO4 cleaved the nascent RNA and then dissociated. Intriguingly, since AGO4 retains the sliced nascent transcript after cleavage (Fig. 1H), the investigators speculate that AGO4 retention of nascent RNA at the target locus could build up a local concentration of silencing proteins at this site, potentially directly recruiting the DNA methyltransferase responsible for de novo DNA methylation.

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References

Havecker ER, Wallbridge LM, Hardcastle TJ, Bush MS, Kelly KA, Dunn RM, Schwach F, Doonan JH, Baulcombe DC. 2010. The *Arabidopsis* RNA-directed DNA methylation argonautes functionally diverge based on their expression and interaction with target loci. *Plant Cell* **22:** 321–334. doi:10.1105/tpc.109.072199

Höck J, Meister G. 2008. The Argonaute protein family. *Genome Biol* **9:** 210. doi:10.1186/gb-2008-9-2-210

Li Q, Gent JI, Zynda G, Song J, Makarevitch I, Hirsch CD, Hirsch CN, Dawe RK, Madzima TF, McGinnis KM, et al. 2015. RNA-directed DNA methylation enforces boundaries between heterochromatin and euchromatin in the maize genome. *Proc Natl Acad Sci* **112:** 14728–14733. doi:10.1073/pnas.1514680112

Liu W, Duttke SH, Hetzel J, Groth M, Feng S, Gallego-Bartolome J, Zhong Z, Kuo HY, Wang Z, Zhai J, et al. 2018. RNA-directed DNA methylation involves co-transcriptional small-RNA-guided slicing of polymerase V transcripts in *Arabidopsis*. *Nat Plants* **4:** 181–188. doi:10.1038/s41477-017-0100-y

Loffer A, Singh J, Fukudome A, Mishra V, Wang F, Pikaard CS. 2022. A DCL3 dicing code within Pol IV–RDR2 transcripts diversifies the siRNA pool guiding RNA-directed DNA methylation. *Elife* **11:** e73260. doi:10.7554/eLife.73260

McCue AD, Panda K, Nuthikattu S, Choudury SG, Thomas EN, Slotkin RK. 2015. ARGONAUTE 6 bridges transposable element mRNA-derived siRNAs to the establishment of DNA methylation. *EMBO J* **34:** 20–35. doi:10.15252/embj.201489499

Mi S, Cai T, Hu Y, Chen Y, Hodges E, Ni F, Wu L, Li S, Zhou H, Long C, et al. 2008. Sorting of small RNAs into *Arabidopsis* argonaute complexes is directed by the 5′ terminal nucleotide. *Cell* **133:** 116–127. doi:10.1016/j.cell.2008.02.034

Qi Y, He X, Wang X-J, Kohany O, Jurka J, Hannon GJ. 2006. Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation. *Nature* **443:** 1008–1012. doi:10.1038/nature05198

Shimada Y, Mohn F, Bühler M. 2016. The RNA-induced transcriptional silencing complex targets chromatin exclusively via interacting with nascent transcripts. *Genes Dev* **30:** 2571–2580. doi:10.1101/gad.292599.116

Sigman MJ, Panda K, Kirchner R, McLain LL, Payne H, Peasari JR, Husbands AY, Slotkin RK, McCue AD. 2021. An siRNA-guided ARGONAUTE protein directs RNA polymerase V to initiate DNA methylation. *Nat Plants* **7:** 1461–1474. doi:10.1038/s41477-021-01008-7

Singh J, Mishra V, Wang F, Huang H-Y, Pikaard CS. 2019. Reaction mechanisms of Pol IV, RDR2, and DCL3 drive RNA channeling in the siRNA-directed DNA methylation pathway. *Mol Cell* **75:** 576–589.e5. doi:10.1016/j.molcel.2019.07.008

Wang F, Huang H-Y, Huang J, Singh J, Pikaard CS. 2023. Enzymatic reactions of AGO4 in RNA-directed DNA methylation: siRNA duplex loading, passenger strand elimination, target RNA slicing, and sliced target retention. *Genes Dev* (this issue). doi:10.1101/gad.350240.122

Ye R, Wang W, Iki T, Liu C, Wu Y, Ishikawa M, Zhou X, Qi Y. 2012. Cytoplasmic assembly and selective nuclear import of *Arabidopsis* Argonaute4/siRNA complexes. *Mol Cell* **46:** 859–870. doi:10.1016/j.molcel.2012.04.013

Zhang H, Lang Z, Zhu J-K. 2018. Dynamics and function of DNA methylation in plants. *Nat Rev Mol Cell Biol* **19:** 489–506. doi:10.1038/s41580-018-0016-z

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